Characterizing Antigen-specific $CD4^+$ T cells Using HLA-DR Oligomers

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ABSTRACT

T cells are activated by the engagement of their surface T cell receptors (TCR) by antigenic peptide bound to major histocompatibility complex (MHC). The success or failure of this TCR to MHC-peptide interaction determines the specificity of T cell action, and thus plays a central role in proper immune function. In this thesis, soluble oligomers of MHC-peptide complex were used to investigate several aspects of the T cell immune response. Soluble fluorescent oligomers of human class II MHC were produced and used to detect $CD4^+$ T cells of particular specificities. The critical parameters of this interaction were determined, and differing behaviors of various T cell clones were observed. The implications of these results are discussed, and MHC oligomers are suggested as powerful tools for the investigation T cell avidity modulation. Using a novel methodology for the analysis of the antigen-specific TCR repertoire which includes identification by MHC oligomers, T cells specific for a peptide derived from influenza were isolated, cloned and sequenced. This pool of sequences was observed to be extremely diverse in both Vβ usage and CDR3 sequence. These results are discussed with regard to the TCR repertoire, structural aspects of TCR/MHC-peptide interaction, and future studies of TCR repertoire analysis. Other studies investigating the triggering mechanism of TCR are summarized and implications of these results for various models of transmembrane activation are discussed. A novel mechanism is proposed involving the reorganization of a receptor oligomer from a specific inhibited state into an uninhibited state. Future directions of research based on the work presented in this thesis are suggested.

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Abbreviations used in this thesis:

APC: Allophycocyanin

APC: Antigen presenting cell

BCR: B cell receptor

BSP: Biotin-ligase substrate peptide

CFSE: Carboxyfluorescein diacetate succinimide ester

DR1: HLA-DR1

DTT: 1,4-dithiothreitol

FITC: fluorescein isothiocyanate

HABA: 2-hydroxyazobenzene-4'-carboxylic acid

HLA: Human leukocyte antigen

IFN-γ: Interferon-gamma

IL-2: Interleukin-2

mAb: Monoclonal antibody

MHC: Major Histocompatibility Complex

PBMC: Peripheral blood mononuclear cells

PBS: Phosphate-buffered saline

PE: R-phycoerythrin

SA: Streptavidin

SA-PE: Streptavidin-phycoerythrin conjugate

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TCR: T cell receptor

βME-SO_3^-: 2-mercaptoethanesulfonic acid

I. Introduction: The role of T cells in the Immune System

I. A. Introduction

Vertebrates have developed a specialized system, the immune system, to recognize and respond to invading pathogenic microorganisms. In this chapter, I will introduce the major branches of the immune response, discuss the source of antigen-binding diversity in the adaptive immune system, examine the presentation of peptides presented by Major Histocompatibility Complex (MHC) molecules to T cells, review mechanisms of T cell activation, and evaluate techniques for the identification of antigen-specific T cells. For further discussion of these and other topics in immunology I suggest the reader consults one of several available textbooks on the subject (Goldsby et al 2000; Janeway & Travers 2001).

I. B. A brief introduction to the immune system

I. B.1 Innate Immunity

One of the most important strategies of the mammalian immune system is the use of multi-layered and potentially redundant defenses, each of which must be separately eluded for an invading pathogen to establish a secure foothold inside the host. The innate immune response consists of mechanisms that identify and clear pathogens according to a limited set of relatively non-specific rules. Included within the innate immune system are the skin and mucosa which exclude pathogens based on their extra-organismal location. Also included are local environmental factors such as gastric pH, body temperature, and oxygen tension which make certain pathways of entry less hospitable. If an invading

organism is able to navigate these primary defenses, they encounter some of the more complex mechanisms of the innate immune response based on the recognition of particular lipids and polysaccharides normally absent in vertebrates and known as pathogen-associated molecular patterns (PAMPs). Examples of such structures include lipopolysaccharide, found in most gram-negative bacteria, peptidoglycan, providing the framework of most bacterial cell walls, and zymosan, a carbohydrate component of yeast cell walls (Aderem & Ulevitch 2000). Cells such as macrophages, neutrophils and monocytes attack pathogens by endocytic and phagocytic mechanisms triggered through cell-surface receptors which are not well understood but are a hot topic of current immunological research. Foremost among these receptors are a family of Toll-like receptors (TLRs) which are reviewed in (Akira et al 2001; Medzhitov & Janeway 2000). A group of serum proteins termed complement react with potentially foreign targets, and either lyse them directly or mark them for destruction by phagocytosis or other cellular mechanisms (reviewed in (Song et al 2000)). Finally, the innate response recognizes the footprint of infection by identifying local tissue damage and triggering the inflammatory response. Soluble factors released by dendritic cells in reponse to necrotic or apoptotic damage recruit virtually every kind of immune cell to the region, causing a massive localized immunological bombardment, which hopefully clears the offending pathogen with minimal damage to the local area.

I. B.2 The Strategy of Adaptive Immunity

If a pathogen successfully evades the forces of the innate immune response, it may still be identified by the more scrupulous adaptive immune response. The adaptive

immune system maintains constant surveillance of all cells and fluids in the body. It screens any protein encountered anywhere in the body and judges whether it signals infection and requires response.

To accomplish this goal, the adaptive immune response needs the capability to bind foreign proteins (antigens) of which it has no previous knowledge. This feat is accomplished by surveillance clones (B and T lymphocytes), each carrying clonotypic antigen-binding proteins on their surface with randomly mutagenized binding sites. However, it is very important that these randomized proteins not bind to any of the proteins native to the host (so-called self-proteins or self-peptides). Accordingly, during development of the individual, clones are screened against self-peptides and self-reactive clones are deleted from the repertoire. This leaves the adaptive immune system with a repertoire of cells specific for unknown non-self proteins. A byproduct of this method is that most surveillance clones will never meet a cognate antigen, if indeed a cognate antigen even exists. Therefore, those that do encounter antigen within the hosts' lifetime become especially useful to the immune system in case the same pathogen ever invades again. In anticipation of this possibility, any clone which becomes activated will proliferate, and some of the resulting progeny will be maintained as memory clones circulating the immune system at a higher frequency than previously. This provides the immune system a rapid and effective response to re-infection, and is the well-known phenomenon of "immunological memory," providing the basis for prophylactic vaccination.

I. B.3 Generation of the Antigen-specific Repertoire

As yet a further example of the multi-layered defenses of the immune system, the above strategy is accomplished in the adaptive immune system by two types of antigen-binding proteins on two surveillance cell types: immunoglobulins (antibodies) on B cells and T cell receptors on T cells. Figure I.1 shows a schematic of the two cells bearing their respective cell-surface receptors, B cell receptor (BCR) and T cell receptor (TCR). The antigen-binding unit of the BCR is the immunoglobulin protein (Ig). At the end of each arm of the Y-shaped Ig is a copy of the antigen-binding site formed by the heavy-chain subunit paired with either the κ or λ light-chain. Once activated, a B cell secretes a soluble version of its immunoglobulin capable of mediating a variety of functions on different cells throughout the immune system. The antigen-binding site of the TCRαβ molecule is formed at the top of the TCRα and TCRβ chains. The antigen-binding site of the more rarely found TCRγδ molecule is formed at the top of the TCRγ and TCRδ chains (TCRγδ is not shown here looks very similar to TCRαβ). While Ig binds directly to proteins in solution, in a virus, or on a cell surface, TCR binds to peptides (derived from any of those sources) carried by Major Histocompatibility Complex molecules found on antigen-presenting cells (APCs).

Immunoglobulins and TCRs generate the vast diversity of their antigen-binding repertoire by several shared mechanisms: permutational diversity achieved by combinations of a limited number of gene segments which together form the binding domain (combinatorial diversity), flexibility of the splice points between the chosen gene segments (junctional flexibility), random addition and deletion of nucleotides at one of the gene-segment junctions (N-region diversity), and random mutation of nucleotides within specific hot spots of the gene (somatic mutation, on immunoglobulin genes only).

Table I.1 summarizes the number of gene segments and activity of these mechanisms on the various genes which make up the antigen-binding proteins Ig, TCRαβ, and TCRγδ. The resulting repertoires have been estimated to contain more than 10^{10} members each (Goldsby et al 2000).

As an example of the gene rearrangement, Figure I.2 depicts the rearrangement of the TCRβ-gene locus. The first rearrangement to occur is the D-to-J joining reaction, followed by V-to-DJ rearrangement. The fully rearranged gene is transcribed into pre-messenger RNA, spliced and translated into polypeptide, which is co-translationally translocated into the endoplasmic reticulum where it folds, pairs with TCRα and is glycosylated. Nearly all of the antigen-binding diversity arises from three loops, named complementarity determining regions, or CDR1, CDR2, and CDR3, on the top of both the TCRα and TCRβ chains, and similarly on the Ig heavy and light chains. CDR1 and CDR2 are contained within, and therefore determined by, Vα, Vβ, V_H or V_L. CDR3 is located at the V-(D)-J junction and is therefore the most diverse region, its diversity arising from junctional diversity and N-region diversity as well as from permutational diversity. In all TCR-MHC structures studied so far, CDR1 and CDR3 contact both MHC and MHC-bound peptide, while CDR2 contacts only MHC (Hennecke & Wiley 2001). In antibodies, all three regions can directly contact antigen (Janeway & Travers 2001).

I. C. T cells and the interaction of TCR with MHC-peptide complex

I. C.1 Introduction to T cells and APCs

TCR molecules on T cells accomplish the critical goal of self/antigen determination, but they are effective only if the antigen-presentation machinery presents a representative and reliable sampling of peptides. Thus, a large portion of the responsibility for thorough surveillance falls on the antigen-presenting cells (APCs). APCs have divided this job into two categories, intracellular and extracellular sampling, and have specialized machinery to deal with each type. In nearly all cells of the body, cytoplasmic proteins are sampled by degradation via the proteasome complex, cathepsins or other proteases (reviewed in (Rock & Goldberg 1999)), and are transported into the ER by the TAP membrane channel, loaded onto class I MHC proteins by specialized machinery, and finally presented on their cell surface (see (Pamer & Cresswell 1998) for a review of these processes). The job of extracellular surveillance falls in the hands of a specialized set of APCs, made up primarily of macrophages, B cells, and dendritic cells. These cells circulate throughout the organism and phagocytose, endocytose and pinocytose proteins, particles, viruses and microbes by a variety of mechanisms (including the PAMP-receptors of the innate immune system and the BCR on B cells, reviewed in (Watts 1997)), degrade proteins in their lysosomes (reviewed in (Villadangos & Ploegh 2000)), load the resultant peptides onto class II MHC proteins in endosomes by a specialized machinery (reviewed in (Busch et al 2000)), and present them at their cell surface for examination by T cells.

The cellular response required to combat intracellular and extracellular infection is quite different, and T cells are divided into two groups in order to respond properly to class I and class II MHC proteins. Antigenic (foreign) peptides bound to class I MHC molecules indicate that the presenting cell contains foreign peptides in its cytoplasm,

signaling infection. In most circumstances, the appropriate response is to kill such infected cells. In contrast, antigen peptides bound to class II MHC molecules indicate that an APC has encountered a foreign invader somewhere in the body. These cells should not be killed and, in fact, may be valuable to help the immune system locate the pathogen. As befits this logic, T cells restricted by (reactive with) class I MHC proteins are cytotoxic and bear the cell-surface glycoprotein CD8 ($CD8^+$ T cells) which binds class I MHC proteins, whereas T cells restricted by class II MHC proteins are non-cytotoxic, and bear the cell surface marker CD4 ($CD4^+$ T cells) which binds class II MHC proteins. $CD4^+$ are alternately termed helper T cells. It should be noted that there exist exceptions to the above rules, and that a subset of $CD4^+$ T cells appear to have some cytotoxic activity (Norris et al 2001; Porakishvili et al 2001; Suni et al 2001). The role of these cells is not well understood.

I. C.2 MHC allelic polymorphism

Every individual carries a set of MHC alleles inherited from each parent. In humans, these genes are carried on chromosome 6 and are termed HLA for Human Leukocyte Antigen, and in mice they are termed H-2 for group II histocompatibility antigens. Humans carry HLA-A, HLA-B, and HLA-C class I MHC genes, and HLA-DR, HLA-DP and HLA-DQ class II MHC genes. Mice carry H-2K, H-2D, H-2L, Qa and Tla class I MHC genes, and H-2 IA and H-2 IE class II MHC genes. Furthermore, MHC genes are highly polymorphic within all mammalian species. For example, within HLA-DR (the most highly expressed human class II MHC protein) there approximately 350 different alleles, which can be grouped into sixteen different families. The set of class I

and class II MHC alleles carried by an individual is referred to as his/her haplotype. The gene product of HLA-DRA*0101 and HLA-DRB1*0101, commonly termed HLA-DR1 or DR1, is carried by 10-20% of the human population and is the MHC studied in this thesis work.

I. C.3 Peptide binding to MHC molecules and recognition by TCR

The role of MHC molecules in this immuno-recognition process is to bind a variety of peptides and to present them for examination to T cells. Figure I.3 shows examples of the two classes of MHC molecules, class I and class II. The modular structure of the two classes is similar, although the domain connectivity is different (Fig. I.3a,b). Each protein is composed of side-by-side immunoglobulin-superfamily domains (beta-sheet sandwiches) topped by a beta sheet bearing a pair of alpha helices. The peptide binding groove is formed between the alpha helices with the beta sheet as the bottom.

Class I MHC molecules bind peptides of 8-10 residues primarily based on their N- and C- terminal residues. These residues are buried into binding pockets at either end of the groove, and the intermediate residues are bulged up in the middle of the groove, available for examination by TCR (Fig. I.3c). The peptide binding motif of nearly all human and murine class I alleles is well understood, and protein sequences can be straightforwardly searched for class I binding peptides (Rammensee et al 1995).

In contrast, class II MHC molecules have an open-ended groove with several distinct pockets for side-chains (P1, P4, P6, P9). These pockets determine the peptide-binding motif of class II MHC molecules. In the case of HLA-DR1, P1 is the most

important pocket for determining specificity, binding large aliphatic or aromatic residues (Fig. I.3d). Peptide binding prediction algorithms for class II MHC molecules (Hammer et al 1994; Southwood et al 1998; Sturniolo et al 1999) have been less successful than for class I MHC molecules, but can nonetheless be an invaluable guide when attempting to identify antigenic peptides. The peptide residues not buried in these pockets are exposed and therefore available for examination by T cells (Fig. I.3d).

I. C.4 The transmembrane signaling mechanism of T cells

The engagement of cell-surface T cell receptor by cognate MHC-peptide complex on the surface of an adjacent APC triggers activation of the T cell. We have separately reviewed the activation mechanism of T cells (Cochran et al 2001a). I will briefly outline the relevant issues and comment on their importance in this thesis work (Cameron et al 2001c; Reth 2001).

Several transmembrane signaling mechanisms are depicted in Figure I.4. In the first mechanism (Fig. I.4a), typical of seven transmembrane span receptors that activate G-proteins (Wess et al 1997), ligand binding induces a structural change in the receptor that can be sensed by effector proteins within the cell. In another mechanism (Fig. I.4b), binding of a multivalent ligand induces receptor co-localization. This mechanism is typical of receptor tyrosine kinases, in which receptor clustering facilitates transphosphorylation by the cytoplasmic kinase domains (Hubbard & Till 2000). In a third mechanism (Fig. I.4c), ligand binding induces a rearrangement of a receptor oligomer into a specific activating conformation. One example of this mechanism is the

bacterial aspartic acid receptor, in which ligand binding induces a helix reorganization that activates receptor-associated cytoplasmic signaling proteins (Falke et al 1997).

The transmembrane signaling mechanism utilized by the TCR on T cells remains controversial (see Cochran, et al., 2001 for a review). Research on T cells until the 1980s focused on genetic and cellular identification and analysis of T cell immunity. In 1982, Jonathan Lamb and coworkers demonstrated activation of influenza-specific T cell clone (HA1.7) by adding chemically synthesized peptide (Ha) to B cells (Lamb et al 1982), capping decades of work demonstrating the interaction between MHC-peptide complex and TCR. But molecular analysis of T cell activation was hindered by the inability of researchers to produce soluble ligand for TCR. To circumvent this problem, researchers spent the next decade studying T cell activation by using anti-TCR antibodies (Kappler et al 1983; Kaye et al 1983; Yoon et al 1994), and chimeric TCRs carrying unrelated extracellular domains (Irving & Weiss 1991; Letourneur & Klausner 1991; Romeo & Seed 1991). These studies combined to show that intermolecular crosslinking of TCR cytoplasmic domains is sufficient to activate some (but not all) signaling pathways, including phosphorylation of tyrosines on the cytoplasmic tails of the CD3 subunits, recruitment of tyrosine kinases, and Ca^{++} mobilization.

In the 1990s the technology to produce soluble versions of some MHC and TCR molecules enabled a detailed molecular analysis of this interaction. Biochemical analysis of the revealed the weak ($\sim 10^{-4}$-10^{-6} M) and short-lived ($t_{½}$ ~1-100 sec) nature TCR/MHC-peptide interactions (Crawford et al 1998; Davis et al 1998). Studies utilizing soluble monomers or oligomers of MHC-peptide complexes to trigger T cells have thus far suggested that $CD4^{+}$ T cells are activated by receptor dimerization

(Cochran et al 2000) or trimerization (Fig. I.4b) (Boniface et al 1998), whereas $CD8^+$ T cells may be activated by MHC-peptide monomers (Delon et al 1998; Sykulev 1996). Although the monomer-activation results appear to suggest receptor structural change as the transmembrane signaling mechanism (Fig. I.4a), CD8 has been suggested to play a key role in the activation by monomers (Delon et al 1998), suggesting a mechanism similar to the receptor co-localization mechanism (Fig. I.4b) but utilizing two different receptors rather than two identical ones. In further support of the receptor co-localization model of T cell activation, structural analysis of a single TCR bound to MHC containing peptides of varying degrees of antigenicity (Ding et al 1999) failed to show any allosteric changes in TCR which might trigger intracellular events. It remains difficult to conclusively rule out receptor rearrangement as a mechanism of T cell activation, although results from our lab indicating that MHC dimers in different orientations are similarly capable of activating $CD4^+$ T cells (Cochran et al 2001b) argue against this model. We recently proposed a variant of this model in which multi-valent engagement of a pre-formed TCR oligomer disrupts a specific inhibited conformation (Cameron et al 2001c). This model will be discussed further in Chapter V.

Another approach to the study of T cell activation has been the use of confocal microscopy to study the real-time molecular organization of the T cell-APC contact region. Recent studies have shown a large-scale molecular segregation of proteins into distinct central and peripheral regions within ten minutes of initial cellular conjugation (Fig. I.5), termed c-SMACs and p-SMACs by one group (Monks et al 1998), and collectively labeled an immunological synapse by another (Grakoui et al 1999). This phenomenon has lead to the proposal of another type of T cell activation mechanism

based on the segregation of regional co-localization of various proteins based on ligand interactions, membrane domain localization, and steric crowding (van der Merwe et al 2000). The role that these large-scale molecular reorganization events play in T cell activation relative to the receptor oligomerization models described above remains controversial (see (Cochran et al 2001a) for a discussion), although recent work suggests that TCR triggering occurs upstream of molecular segregation (Zaru et al 2002).

Despite the remaining confusion regarding the transmembrane signaling mechanism of TCR, one result remains clear: TCR clustering (by either monoclonal antibodies or soluble oligomers of MHC-peptide) can trigger the some degree of activation in either $CD4^+$ or $CD8^+$ T cells (Boniface et al 1998; Cochran et al 2000; Irving & Weiss 1991; Letourneur & Klausner 1991; Yoon et al 1994). This is an important point for the work described in this thesis.

I. D. Placing the work of this thesis into context

The work presented in this thesis hinges on the use of a recently developed reagent for the investigation of T cells, commonly called MHC tetramers, although in this work most often termed MHC oligomers. The most common use of MHC tetramers is to identify T cells of particular antigen-specificity, often by clinical researchers investigating particular diseases. We have been among the first to use class II MHC oligomers on $CD4^+$ T cells, and have used them in collaborations with several clinical researchers, as well as for probing activation characteristics of T cells. I will begin by reviewing techniques for the identification of antigen-specific T cells and finish with a brief discussion of the work presented in the following chapters.

I. D.1 Techniques of antigen-specific T cell identification

The identification and enumeration of antigen-specific T cells is extremely important in the study of diseased states in order to understand what the immune system is doing right, what it is doing wrong, and, potentially, how we might be able to help it work differently. For example, the difference between HIV-infected patients with progressive and non-progressive pathologies has been proposed to reside in the presence or absence of T cells with particular reactivities (Cohen 2001; Rosenberg et al 1997). Autoimmune diseases are widely thought to be aberrant recognition of self-antigens by the immune system (such as the hypothesized recognition of myelin basic protein in multiple sclerosis (Martin et al 1992)), although so far no such antigens have been incontrovertibly identified.

A variety of methods currently exist for the identification of antigen-specific T cells and are summarized in Table I.2. Several of these methods depend critically on the response of the T cells to activation. They fall into two categories representing the two hallmarks of T cell activation: the secretion of soluble factors, and cell proliferation. Depending on their subset, T cells secrete a variety of soluble proteins called cytokines when activated. These cytokines can be measured in bulk by enzyme-linked immuno-sorbent assay (ELISA). The actively secreting cells themselves can be directly detected by disrupting vesicle traffic with a metabolic inhibitor, fixing, permeabilizing and fluorescently staining the cells with anti-cytokine antibodies (intracellular cytokine staining, (Prussin & Metcalfe 1995)). Alternatively, the cytokines can be captured either on the cell surface (surface-capture cytokine staining, (Manz et al 1995)) or in the local

environment (ELISPOT assay, (Czerkinsky et al 1988)) using combinations of anti-cytokine antibodies either fluorescently labeled or enzyme-conjugated.

Bulk assay of lymphocyte proliferation has been routinely measured for several decades by the incorporation of tritiated thymidine in dividing cells (Strong et al 1973). More recently, a method utilizing limiting dilution of T cells followed by expansion and tritiated thymidine incorporation (limiting dilution analysis, (Merkenschlager et al 1988)) was developed to estimate the number of responding clones in the original mixed population. Finally, the ultimate proof of antigen-specific T cells is achieved by establishing T cell clones by the isolation of single cells followed by massive expansion (Fathman & Fitch 1982), and subsequent demonstration of their functions and specificity directly.

However, none of the above methods can directly identify antigen-specific T cells, independent of their functional capacity. This is critical, as phenomena that might obscure or blunt T cell functions, including T cell tolerance, exhaustion, suppression, and anergy, have become increasingly important aspects of immunological research.

Other methods to detect antigen-specific T cells rely on the skewing of the TCR repertoire by monoclonal or oligoclonal expansions. Large expansions of T cells within TCR Vβ families can be detected using Vβ-specific monoclonal antibodies (Diu et al 1993). Similar data can be derived using semi-quantitative PCR utilizing distinct sets of Vα- or Vβ-specific oligonucleotide primers, combined with fine analysis of CDR3 lengths by high-resolution electrophoresis (spectratyping, (Pannetier et al 1993)). Finally, dominant clones can be visualized by PCR amplification, hybridization with similar but non-identical excess of carrier DNA, and non-denaturing DNA

electrophoresis (heteroduplex analysis, (Wack et al 1996)). These methods ignore low-frequency T cells, and identify T cells only by means of statistical comparison with typical distributions, rather than by direct identification based on specificity.

Several years ago a novel technique of antigen-specific T cell detection termed "MHC tetramer analysis" was developed by Altman and colleagues (Altman et al 1996) which has proved extremely popular in the analysis of $CD8^+$ T cells. MHC tetramers are soluble versions of the MHC molecules, biotinylated at the C-terminus of one of the subunits, and subsequently oligomerized using fluorescent streptavidin. Although TCR/MHC-peptide interactions are typically weak (~10^{-4}-10^{-6} M) with short half-lives (~1-100 sec) (Crawford et al 1998; Davis et al 1998), oligomers of MHC-peptide should have relatively high avidity and slow off-rates. Making these oligomers fluorescent turns them into convenient and sensitive probes of the TCR/MHC-peptide interaction. The technique of MHC tetramer staining of T cells has enabled the direct identification of antigen-specific $CD8^+$ T cells in principle based on TCR specificity, and neither T cell function nor TCR sequence (McMichael & O'Callaghan 1998). The success of this technique (Callan et al 1998; Goulder et al 2000; Kuroda et al 1998; McHeyzer-Williams et al 1996; Moser et al 2001; Murali-Krishna et al 1998; Yee et al 1999) has led to the establishment of a NIH-sponsored facility that produces class I MHC tetramers and provides them free of charge to researchers around the world (see www.niaid.nih.gov/reposit/tetramer/index.html). Only recently has this technology been extended to the use of class II MHC tetramers to study $CD4^+$ T cells (Cameron et al 2001a; Crawford et al 1998; Kwok et al 2000; Meyer et al 2000; Novak et al 1999; Savage et al 1999).

I. D.2 Overview of thesis

This thesis describes the development and use of soluble fluorescent streptavidin-based oligomers of HLA-DR1 to identify and investigate antigen-specific $CD4^+$ T cells.

Chapter II describes the production of soluble fluorescent streptavidin-based oligomers of the class II MHC HLA-DR1 and their use on $CD4^+$ T cell clones. The experimental behavior of these reagents is characterized using fluorescent microscopy, biochemical experiments, and various inhibitors of cellular functions. In this chapter we conclude that the staining of some $CD4^+$ T cells requires a cellular response and is not, therefore, function-independent, as the theory of MHC tetramers suggests. Jennifer R. Cochran contributed to this work, and Yassine-Diab Bader in the lab of Dr. Rafick Pierre-Sekaly provided a valuable short-term T cell line for this work. This chapter was published in January 2001 as an article in the Cutting Edge section of the Journal of Immunology (Cameron et al 2001a).

Chapter III describes in greater detail the method for production of biotinylated DR-peptide complex from *E. coli* and compares it to production from insect cell culture. DR oligomers are used to stain various $CD4^+$ T cell clones and polyclonal lines and the temperature-dependence of staining these lines is analyzed. Finally, the use of these reagents as probes for T cell avidity modulation is discussed. Valuable reagents and advice were contributed to this work by Philip J. Norris and Eric S. Rosenberg at Mass. Gen. Hospital, Alka Patel and Lucy R. Wedderburn at the Institute for Child Health in London, Corinne Moulon at Dictagene in Switzerland, and Elizabeth D. Mellins at Stanford University. This chapter has been submitted to the Journal of Immunological

Methods for inclusion in a special issue devoted to MHC tetramers slated for publication in early 2002 (Cameron et al 2001b).

Chapter IV describes the use of MHC oligomers and IFN-γ surface-capture staining to identify $CD4^+$ T cells specific for a peptide derived from influenza in samples of peripheral blood. The repertoire of TCRβ proteins utilized by these cells is found to be extremely broad, and the importance of these findings and directions for future research within the study of the TCR repertoire are discussed. This work was a close collaboration with George B. Cohen at Mass. Gen. Hospital, and has been prepared in the form of a manuscript for submission in the near future.

Chapter V describes other research in this laboratory to which I have contributed and discusses the implication of results for current models of T cell triggering. These studies (Cochran et al 2000; Cochran et al 2001b), performed primarily by Jennifer Cochran, utilized soluble oligomers of MHC-peptide complexes at precise valency to probe the triggering mechanism of $CD4^+$ T cells. TCR dimerization was found to be the critical factor determining T cell triggering. The relative orientation of clustered TCR appeared to be irrelevant to the signal transduced, and TCR activation increased with shortened inter-TCR distances. I discuss the relation between these results and various models of transmembrane signal transduction. In particular, I propose a novel mechanism of signal transduction in which the inhibitory conformation of an oligomeric receptor is disrupted by multivalent engagement. A portion of this chapter was published as a Letter in Trends in Immunology (Cameron et al 2001c).

Chapter VI summarizes the conclusions of this thesis and discusses possible future directions of research based on the work presented here.

The Appendices include detailed protocols used or developed during my graduate career, tables of content for my research notebooks, and notes on various DNA constructs and cell transfectants.

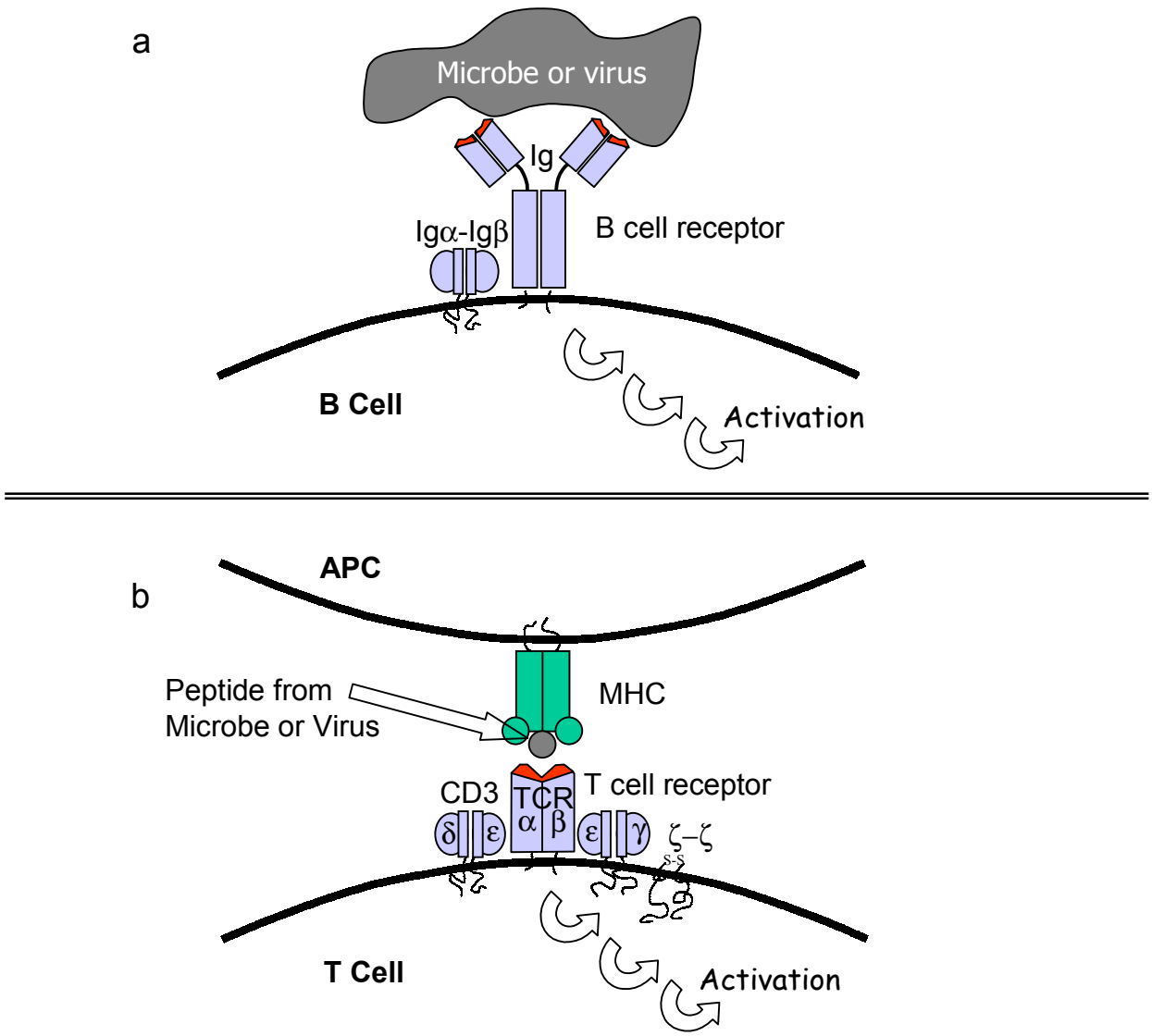

Figure I.1. *Antigen receptors of the adaptive immune response*. Schematic diagrams of the subunit composition of B cell Receptor and T cell receptor and their cognate ligands. The precise stoichiometry of subunits in either receptor is unclear. The antigen-binding sites of Ig and TCRαβ are composed of CDR1, CDR2 and CDR3 loops and are highlighted in red at the top of each protein. (a) The B cell receptor is shown engaged with a microbe or virus. B cell receptor is composed of one membrane-anchored immunoglobulin molecule (Ig) responsible for binding antigen associated with Igα-Igβ heterodimer responsible for triggering intracellular signaling cascades. (b) The T cell receptor is shown binding to MHC-peptide complex presented by an adjacent antigen presenting cell (APC). T cell receptor is composed of one TCRαβ heterodimer responsible for antigen-binding associated with ζ-ζ homodimer and CD3δ-ε and/or CD3γ-ε heterodimers, collectively responsible for triggering intracellular signaling cascades.

Table I.1. *Sources of diversity in the B cell and T cell repertoires*

	# of gene segments			Combini-torial	Junctional Flexibility	N-region Diversity	Somatic Mutation
Ig	V	D	J				
κ light chain	~ 100	-	5	+	+	-	+
λ light chain	~ 100	-	6	+	+	-	+
heavy chain	75-250	30	6	+	+	+	+
TCRαβ							
α chain	46	-	50	+	+	+	-
β chain	48	2	13	+	+	+	-
TCRγδ							
γ chain	6		5	+	+	+	-
δ chain	8	3	4	+	+	+	-

Fig. I.2 *Synthesis and expression of TCRβ*

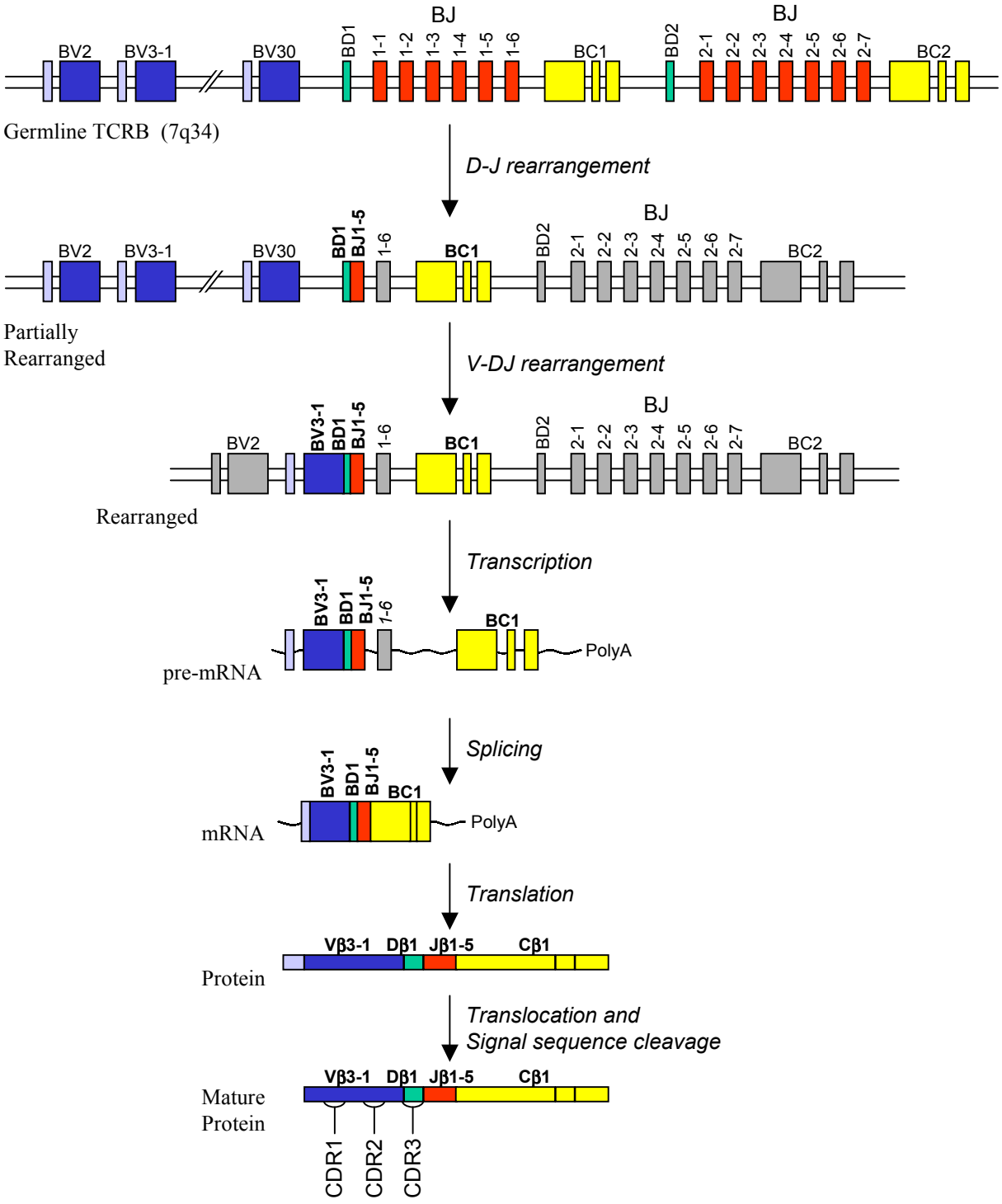

Figure I.2. *Synthesis and expression of TCRβ.* The diversity of the antigen-binding sites of Ig and TCRαβ are generated during somatic rearrangements of the Ig and TCR genes during lymphocyte development. TCRβ rearrangement and expression is shown as an example. In the first step, a D segment is joined to a J segment of its neighboring set. In the second step one of the V segments is joined to the DJ pair. In each case the intervening DNA is excised and discarded. The rearranged VDJ gene is transcribed with the adjacent C segment, spliced, translated into protein, translocated into the ER and paired with a surrogate TCRα chain. If during rearrangement there was an inappropriate frame shift or insertion of a stop codon, the gene product is unproductive and so either further rearrangement at this locus or at the locus site on the other TCRβ gene-bearing chromosome occur. If the rearrangements resulted in a productive VDJ combination, then rearrangements commence at the TCRα locus in a similar manner.

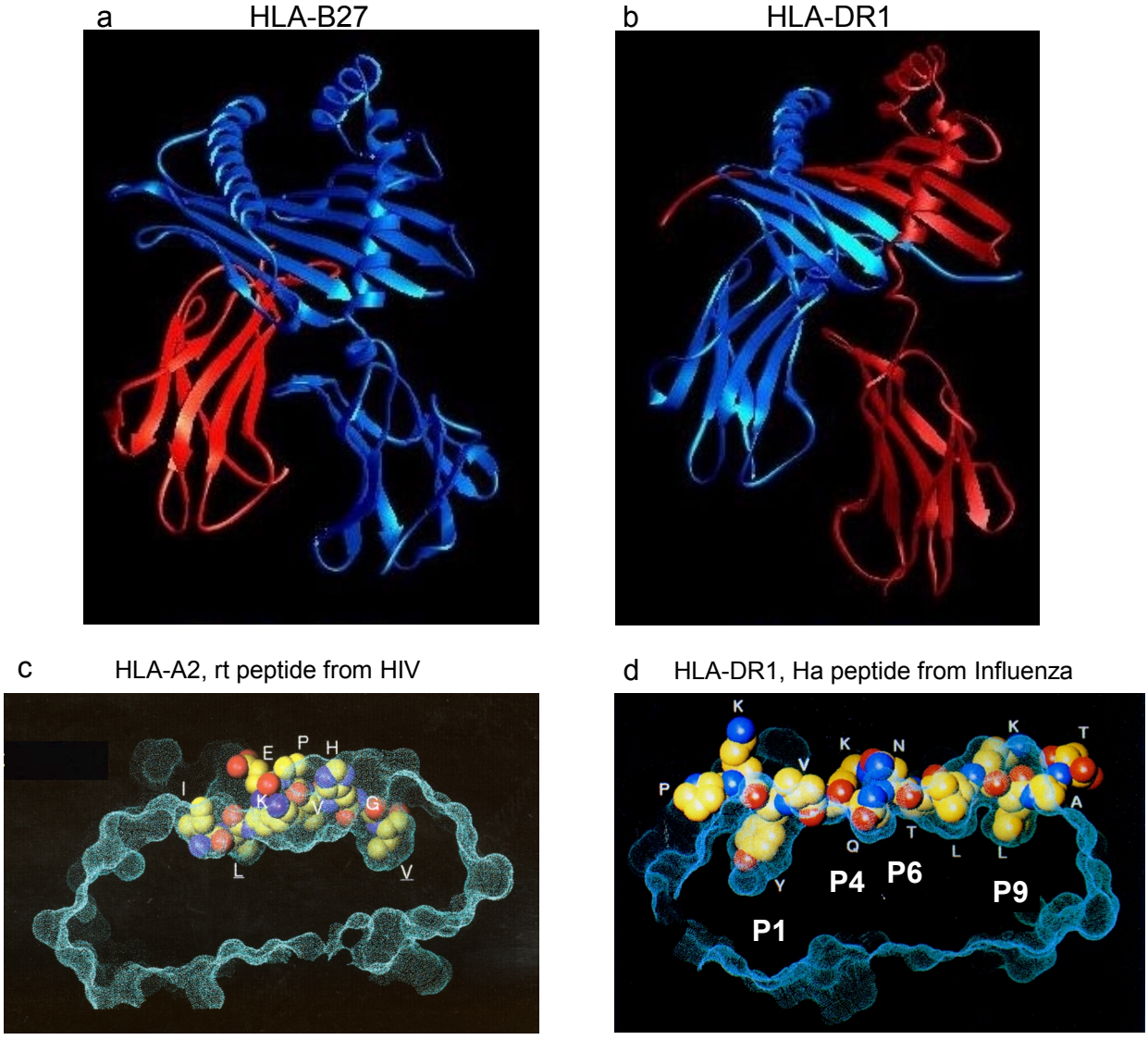

Figure I.3. *Structures of MHC-peptide complexes*. Peptide-binding MHCs are divided into class I and class II proteins. Structures determined by x-ray crystallography of examples from each class are shown. (a,b) The human class I MHC protein HLA-B27 (a) and human class II MHC protein HLA-DR1 (b) are shown without peptide looking down the peptide-binding groove. The lower two domains of each MHC are beta-sheet sandwiches and are members of the immunoglobulin-superfamily of protein domains. On top of these domains sits a beta-sheet, topped by an alpha-helix on either side of the peptide binding groove. In native proteins, transmembrane domains would extend from the C-termini of each MHC at the bottom of the proteins as they are shown here. (c,d) These images are 90 degrees rotated around the vertical axis of the images shown in (a,b). Surface topologies of just the upper peptide-binding domain are shown with space-filled representations of bound peptide. In (c) the class I MHC HLA-A2 is shown bound to a peptide derived from the reverse transcriptase protein of HIV. Note that the terminal peptide residues are buried in the MHC while the intervening residues are bulged upwards available for contact by T cell receptor. In (d) HLA-DR1 is shown bound to a peptide derived from the hemagglutinin protein of influenza. In contrast to the class I MHC-peptide complex, in the class II MHC the peptide binding groove is open-ended and the terminal peptide residues appear to make minimal contact with the MHC. Instead, peptide binding is determined by residues in several distinct pockets along the groove, labeled P1, P4, P6 and P9, and resides of the peptide in between these pocket-bound residues are available for contact by T cell receptor. Panels (c,d) are reproduced from Stern & Wiley, Structure, 1994.

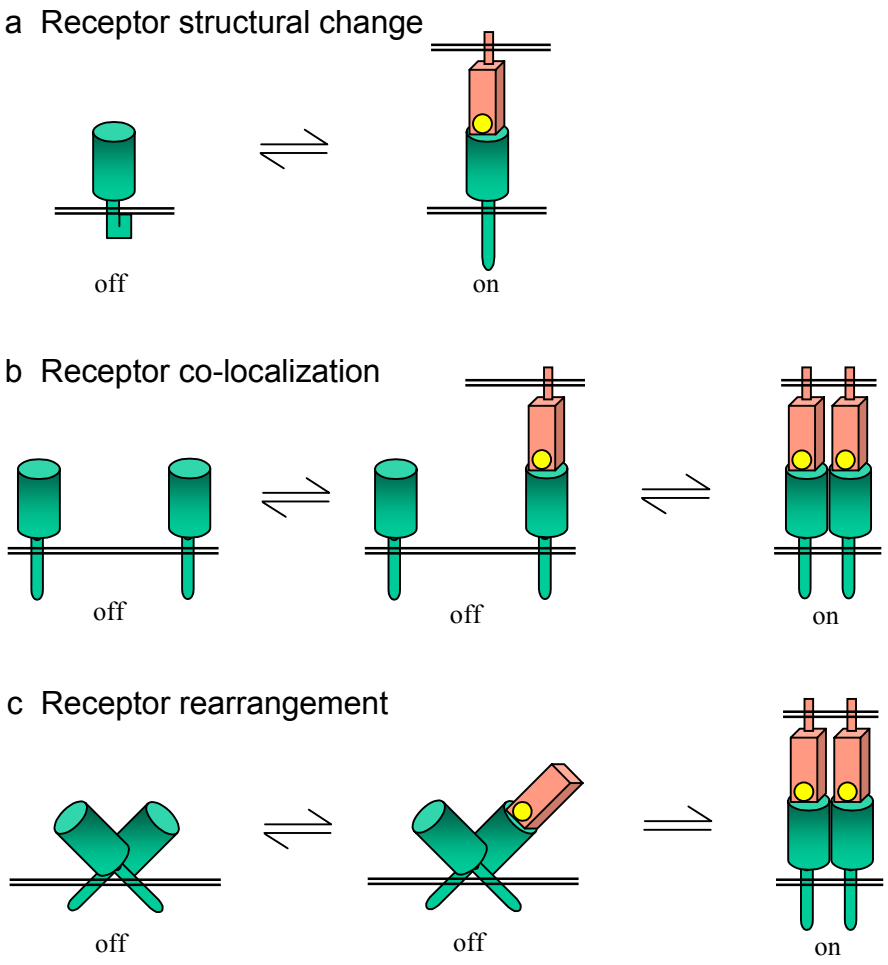

Figure I.4. *Mechanisms of transmembrane signaling.* (a) Ligand-induced conformational change, typified by seven transmembrane span receptors that activate G-proteins. (b) Ligand-induced receptor co-localization, typified by the fibroblast growth factor receptor tyrosine kinase, which is activated by dimerization. (c) Ligand-induced allosteric rearrangement, typified by the bacterial aspartic acid receptor, in which ligand binding induces a helical reorientation.

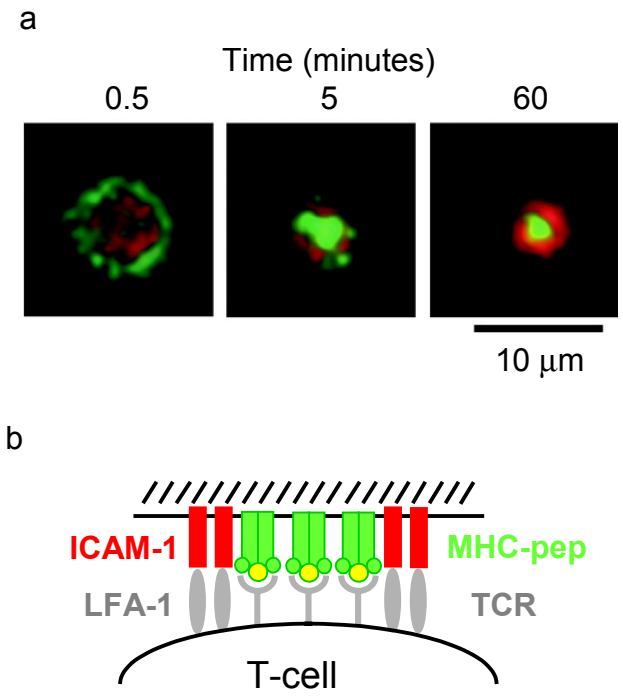

Figure I.5. *Large-scale membrane rearrangements during T cell activation.* (a) Fluorescent microscopy of interaction between a T cell and a supported bilayer carrying labeled MHC-peptide complexes (green) and ICAM adhesion molecules (red). The view is normal to the membrane interface. Image excerpted from Grakoui, Science, 1999 with permission for publication in Cochran, TiBS, 2001. Copyright 1999 American Association for the Advancement of Science. (b) Schematic diagram of the process visualized in (a) with appropriate color coding.

Table I.2. *Methods to Detect Antigen-specific T cells*

Property	Technique	Principle	Notes
Proliferation	Bulk Proliferation Assay T Cell Cloning Limiting Dilution Analysis	*In vitro* stimulation of T cells.	Difficult to determine %-antigen-specific in original sample. Assays all biased for clones which most easily proliferate *in vitro*.
Cytokine Secretion	Intra. Cytokine Staining Cytokine Surface-capture EliSpots	Detects, and Quantitates individual cells which secrete particular cytokines in response to APC-ag stimulation.	Depends on cytokine profile, activation state, and *in vitro* stimulation.
TCR Diversity	TCR Vbeta mAb Spectratyping Heteroduplex analysis	Probe for skewing of the normal TCR repertoire caused by monoclonal or oligoclonal expansion of cells.	Indirect; doesn't identify the specificity of the populations examined. Relatively insensitive. Spectratyping and Heteroduplex analysis are technically challenging.
TCR Specificity	MHC "tetramer" staining	"Tetramers" bind to cell surface TCR and identify ag-specificity directly.	Extremely direct and facile assay. However, reagents difficult to obtain. Requires knowledge of antigenic peptides.

II. Detection of Antigen-Specific $CD4^+$ T cells by HLA-DR1 Oligomers Depends on the T cell Activation State

Class I MHC tetramers have proven to be invaluable tools for following and deciphering the $CD8^+$ T-cell response, but the development of similar reagents based on class II MHC proteins for detection of $CD4^+$ T cells has been more difficult. We evaluated fluorescent streptavidin-based oligomers of HLA-DR1 for use as reagents to analyze antigen-specific human $CD4^+$ T cells. Staining was blocked at low temperatures and by drugs that disrupt microfilament formation and endocytosis. Cell-associated MHC oligomers were resistant to a surface stripping protocol, and were observed by microscopy in intracellular compartments. This behavior indicates that detection of $CD4^+$ T cells using class II MHC oligomers can depend on an active cellular process in which T cells cluster and/or endocytose their antigen receptors. T cells of identical specificity but in different activation states varied greatly in their ability to be detected by class II MHC oligomers.

II.A. Introduction

Tetramers of class I MHC proteins have been shown to bind to antigen-specific $CD8^+$ T cells with high specificity and sensitivity, thus providing a rapid *in vitro* assay for T-cell detection and enumeration that in principle depends on neither the activation nor differentiation state of the T cell (McMichael & O'Callaghan 1998). The ability of class I MHC tetramers to detect low frequency antigen-specific T cells in mixed populations has led to increasing use of these reagents in studies of the immune response (McMichael & O'Callaghan 1998).

In recent reports, MHC tetramer technology has been extended to the class II system (Crawford et al 1998; Kotzin et al 2000; Kwok et al 2000; Savage et al 1999). These reports used relatively high concentrations of tetramer (20 μg/ml), extended incubation times (1-3 hours), and elevated temperatures (22°C or 37°C). A requirement for such conditions would be surprising, given the avidity and fast on-rates expected for the oligomeric MHC-TCR interaction (Davis et al 1998; Eisen et al 1996). This suggests that class II MHC tetramer staining may reflect a more elaborate process than simple association of a soluble reagent with surface receptors.

In the current study, we investigated the utility of oligomers of human class II MHC-peptide complexes in detection of antigen-specific $CD4^+$ T cells. We show by flow cytometry that fluorescent oligomers of DR1 in complex with a peptide from influenza virus specifically stain two DR1-restricted, influenza-specific T cell clones and an antigen-specific polyclonal T cell line, and that the oligomers are internalized efficiently. Treatments that interfere with cytoskeletal rearrangements and endocytosis block class II MHC oligomer staining, showing that an active cellular process is required. Implications of these results for the use of class II MHC oligomers in detection of antigen-specific $CD4^+$ T cells are discussed.

II. B. Materials and Methods

II. B.1 Peptides

Ha [306-318] (PKYVKQNTLKLAT), TT [830-844] [QYIKANSKFIGITEL], A2 [103-114] (VGSDWRFLRGYHQYA), and TfR [680-696] (RVEYHFLSPYVSPKESP) were synthesized using solid-phase Fmoc chemistry, and purified by C18 reverse-phase HPLC. All peptides bind tightly to DR1 with dissociation constants below 100 nM (Zarutskie et al 1999).

II. B.2 Preparation of labeled antibodies and streptavidin.

Murine monoclonal antibodies OKT3 or OKT4 (American Type Culture Collection) purified from hybridoma supernatant or streptavidin (Prozyme) were incubated with ten-fold molar excess FITC (Sigma-Aldrich), succinimidyl 6-(biotinamido) hexanoate (NHS-LC-biotin, Pierce), or N-(6-(biotinamido)hexyl)-3'-(2'pyridyldithio) propionamide (Biotin-HPDP, Pierce), at pH 7.5 for 3 hours at room temperature and isolated by gel filtration using Sephadex G-50 (Pharmacia). Streptavidin was pre-incubated with 2-hydroxyazobenzene-4'-carboxylic acid (HABA, Pierce Chemical Co.) before fluorescent labeling. R-phycoerythrin-conjugated streptavidin was purchased from Biosource, Inc.

II. B.3 Preparation of fluorescent class II MHC oligomers.

Soluble HLA-DR1 peptide complexes carrying an alpha subunit C-terminal cysteine (Cochran et al 2000) were reacted with excess maleimide-oxyethylene-biotin (PEO-maleimide activated biotin) or pyridyldithio-propionamide-biotin (HPDP-biotin, Pierce Chemical Co.) and isolated by gel filtration in phosphate buffered saline (PBS), pH 7.0. Oligomers were formed by stepwise addition of streptavidin-R-phycoerythrin conjugate (SA-PE, Biosource, Inc.). SA-PE from other sources were tested and found to label cells less brightly. FITC-labeled tetramers were isolated by gel filtration using sequential SEC-3000 (Phenomenex) and Superdex 200 (Pharmacia) columns. Streptavidin absorbed to the gel filtration matrix, but the DR-saturated SA-FITC tetramer eluted at its expected molecular weight.

II. B.4 T cell clones and line.

T cell clones HA1.7 and Cl-1 were cultured as described (Cochran et al 2000), and rested six or more days before use. A short-term polyclonal $CD4^+$ T cell line was raised by repeated *in vitro* stimulation of CD8-depleted PBMCs from a DR1-homozygous donor using autologous mitomycin C-treated PBMCs in the presence of 1 μM Ha peptide.

II. B.5 Flow cytometry.

T cells (~10^7/ml) were mixed with DR1-peptide oligomers for 3-5 hrs. at 37°C, chilled for 5 min., supplemented with secondary antibodies for 30 min., and washed twice with cold wash buffer (PBS, 1% fetal bovine serum, 15 mM sodium azide). Inhibitors (stock solutions in DMSO, ethanol or PBS; final concentration of solvent ≤ 0.5%) were pre-incubated with cells in PBS for 1 hour, after which the cells were stained with oligomer as above. In cell surface stripping experiments, chilled and washed cells were resuspended in wash buffer containing 25 mM 2-mercaptoethanesulfonic acid (βME-SO_3^-), incubated for fifteen minutes at 37°C, and then washed twice with cold wash buffer. In inactivation experiments, cells were pretreated overnight in complete medium with peptide or with immobilized OKT3 (5 μg/ml in PBS, 2hrs), and then were stained as above.

II. B.6 Fluorescence microscopy.

Live HA.1.7 T cells were isolated using a Ficoll gradient and incubated with 10 mg/ml FITC-dextran (average MW 10 kDa, Sigma-Aldrich) and 70 μg/ml DR1-Ha SA-PE oligomer, or 35 μg/ml SA-PE alone, for three hours at 37°C, seeded onto cold Cell-Tak (Becton Dickinson Labware) coated glass coverslips, washed, fixed, mounted in Fluoromount-G

(Electron Microscopy Sciences), and visualized using a DeltaVision digital deconvolution microscope system (Applied Precision Inc.).

II. B.7 Dynamic light scattering.

Measurements were made at 22°C using a Protein Solutions DynaPro-MS/X dynamic light scattering instrument. Protein samples were filtered through 0.2 micron spin filters (Costar) before analysis. All samples were measured at two different protein concentrations and with identical results. Molecular weight equivalents (Da) were estimated from hydrodynamic radii (R_H, nm) using an empirical model for globular proteins, log MW = 2.426 log (1.549xR_H), as recommended by the manufacturer.

II. C. Results

II. C.1 DR1 oligomers detect antigen-specific $CD4^+$ T cells.

The oligomerization strategy used in this work relies on biotin covalently coupled to a cysteine residue at the C-terminus of the HLA-DR1α subunit (Cochran & Stern 2000), with subsequent oligomerization using streptavidin. Soluble DR1-peptide complexes, folded *in vitro* from subunits expressed in *E. coli* (Frayser et al 1999), were biotinylated at the introduced cysteine with >90% efficiency, using a maleimide reagent carrying biotin at the end of a 29 Å hydrophilic linker (Fig. II.1*a*). The DR1-peptide complexes were resistant to SDS-induced chain dissociation at room temperature, indicating quantitative peptide loading (Fig. II.1*a*). For routine use, biotinylated DR1-peptide complexes were oligomerized with streptavidin conjugated to R-phycoerythrin. Such DR1-SA-PE oligomers carrying the Ha peptide exhibited antigen-specific binding to two DR1-restricted, Ha-specific, T cell clones, HA1.7 (Lamb et al

1982) and Cl-1 (De Magistris et al 1992) (Fig. II.1*b,c*). Oligomers carrying unrelated peptides showed no significant binding (Fig. II.1*b,c*; A2 and TfR traces). To confirm the specificity of oligomer binding we analyzed a series of dilutions of the HA1.7 clone mixed into non-specific peripheral blood mononuclear cells (PBMCs). Monocytes present in the PBMC mixture exhibited non-specific oligomer binding, but could be distinguished by their $CD4^{med}$ phenotype (Fig. II.1*d*). The fraction of T cells staining with the DR1-SA-PE oligomers correlated closely with the fraction of antigen-specific cells in the mixture, highlighting the specificity of staining (Fig. II.1*d*).

II. C.2 Oligomer staining is observed only under conditions permissive for internalization.

We examined the experimental conditions necessary for oligomer staining of the T cell clones. Both clones required relatively high oligomer concentration (Fig. II.2*a*), with Cl-1 exhibiting saturating staining intensity at >300 μg/ml (~0.6 μM), while for HA1.7, staining did not appear to saturate even at 750 μg/ml (~2 μM). Staining intensity increased with increasing temperature for both clones (Fig. II.2*b*). At 4°C, the temperature usually employed for antibody staining, no staining was observed for either clone. The staining signal developed slowly, and continued to increase for at least five hours after addition of oligomer (Fig. II.2*c*). These concentration, temperature, and time requirements for efficient staining with these reagents are consistent with those described by other researchers using MHC class II oligomers (Crawford et al 1998; Kotzin et al 2000; Kwok et al 2000). Our typical staining protocol employs 20-50 μg/ml oligomer reagent for 3-5 hours at 37°C.

The elevated temperature and extended time required for efficient staining suggested that a metabolic process might be involved. We investigated the staining of T cells by DR-SA-

PE oligomers after treatments that interfere with various normal cellular functions (Fig. II.2*d*). Treatments that inhibited conventional T cell signaling pathways, including genistein (Akiyama et al 1987), staurosporine (Tamaoki et al 1986), PP2 (Hanke et al, 1996), and methyl-β-cyclodextrin (Scheiffle et al, 1997), weakly inhibited staining or had no effect. Agents that disrupt endosomal proteolysis, such as chloroquine, NH_4Cl, and monensin, had little or no effect. However, staining was substantially blocked by treatments that interfere with endocytosis and cytoskeletal rearrangements, including the microfilament-disrupting drugs latrunculin A (Spector et al 1989) and cytochalasin D (Goddette & Frieden 1986), the phosphatase inhibitor phenylarsine oxide (Lazari et al 1997), sodium azide (80mM), and paraformaldehyde fixation. T cell receptors are efficiently internalized following engagement by cell-surface MHC-peptide complexes on other cells (Itoh et al 1999; Valitutti et al 1995). Since MHC oligomer staining was blocked by treatments which block endocytosis, we postulated that oligomers might be internalized along with TCR during the staining protocol.

II. C.3 Bound class II MHC oligomers are present in internalized compartments.

To test whether MHC class II oligomers were internalized after binding, we performed fluorescence microscopy on HA1.7 T cells after incubation with DR-SA-PE oligomers. Oligomer staining was detected in intracellular compartments (Fig. II.3*a*, red), co-localized with endocytic compartments as visualized by FITC-dextran (Fig. II.3*a*, green). Cells incubated with SA-PE reagent alone (without MHC) showed normal FITC-dextran internalization but no detectable PE signal (not shown). These results show that MHC class II oligomers are internalized efficiently by T cells.

We used a surface stripping protocol to evaluate the relative contributions of internalized and surface-bound oligomers to the staining signal observed by flow cytometry. A disulfide bond was introduced into MHC tetramers between the DR1 and biotin moiety (DR1-SS-SA-PE), to allow cleavage by the membrane-impermeant reducing agent 2-mercaptoethane sulfonate (βME-SO_3^-). DR1-SS-SA-PE oligomer staining of HA1.7 T cells was not affected by the βME-SO_3^- treatment (Fig. II.3*d*), indicating that cell-associated oligomers were not present at the cell surface. To demonstrate the effectiveness of the stripping protocol, we examined HA1.7 T cells that had been surface-stained with an antibody carrying the cleavable linker (αCD4-SS-bio). In this experiment, staining was limited to the cell surface by incubation at 4°C. Fluorescent streptavidin was efficiently stripped from the –SS-bio antibody by the βME-SO_3^- treatment (Fig. II.3*b*), but not from a non-cleavable biotinylated antibody (Fig. II.3*c*). The insensitivity of the oligomer-binding signal to the βME-SO_3^- surface-stripping protocol, and the lack of surface staining observed by fluorescence microscopy, both indicate that essentially all of the oligomers associated with T-cell staining are present in internal compartments.

One possibility for the correlation between MHC oligomer staining and internalization might be a low valency in preparations of DR1-SA-PE. The actual oligomeric state of MHC-SA-PE oligomers has not been reported. Moreover, physical characterization is difficult due to the large size of the PE moiety (~250 kDa) and heterogeneous crosslinking in commercial preparations of SA-PE. To obtain MHC oligomers with a well-defined valency, we prepared oligomers using SA labeled with FITC. Because several preparations of commercially available SA-FITC conjugates exhibited sub-stoichiometric biotin-binding capacity, we developed a strategy to prevent damage to the biotin-binding sites during FITC labeling, by protection of the biotin-binding sites using the weakly binding biotin analog HABA (see

Methods). DR1-SA-FITC oligomers prepared with this reagent exhibited an average molecular weight (230 kDa) consistent with one SA (60 kDa) and four DR1 (45 kDa), indicating that the desired tetrameric species had been formed (Fig. II.3*g*). Gel filtration analysis gave a similar result (265 kDa, data not shown). By contrast, DR1-SA-PE oligomers exhibited a heterogeneous population distributed around MW 15,000 kDa (Fig. II.3*f*), indicating that they are composed of multiple SA and/or PE moieties; they are either large oligomers or non-covalent aggregates, and properly they should not be referred to as "tetramers." T cell staining by the defined DR1-SA-FITC tetramers (Fig. II.3*e*) was considerably less intense than that observed for the DR1-SA-PE oligomers (Fig. II.3*d*), even after expression of the staining results in terms of numbers of fluorophores bound per cell (data not shown). Nonetheless, the observed staining still required elevated temperature and was insensitive to surface stripping by βME-SO_3^- (Fig. II.3*e*). Thus, the observed association of staining and endocytosis was not due to a reduced valency in the DR1-SA-PE oligomers.

To characterize the internal compartment associated with MHC class II staining, we made use of the differential pH sensitivity of two fluorescent dyes with similar spectral characteristics, FITC and Bodipy-FL (4,4'-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid). FITC conjugates show reduced staining intensity at lower pH (for example exhibiting 60% reduction from pH 7.2 to pH 6.0) (Yguerabide et al, 1994), whereas Bodipy-FL conjugates are insensitive to pH in the range 2-10 (Karolin et al, 1994). We stained HA1.7 T cells with DR1-Ha tetramers labeled with either FITC or Bodipy-FL, and converted the fluorescence intensities to number of tetramers bound per cell, to account for differences in the intrinsic fluorescent yield of the dyes. The curve describing the binding of the FITC-labeled tetramers (Fig II.4a) was significantly lower than that for the Bodipy-FL tetramers (Fig. II.4b).

Addition of NH_4Cl increased the FITC signal to a level comparable to the Bodipy-FL signal (Fig. II.4a, *open squares*), but had no effect on the Bodipy-FL signal (Fig. II.4b*, open squares*). Both the reduced intensity of the FITC signal relative to that for Bodipy-FL, and the NH_4Cl sensitivity of the FITC signal, indicate that MHC tetramers are internalized to acidic compartments.

II. C.4 A polyclonal T cell line contains subpopulations with different requirements for staining.

To test the generality of the observation that internalization is required for staining with MHC class II oligomers, we tested a polyclonal T cell line restricted by HLA-DR1 and specific for the Ha peptide. Oligomer staining experiments were performed in parallel at 37°C and at 4°C (Fig. II.5*a*). At 37°C, 67% of the total polyclonal cell population exhibited DR1-SA-PE staining (PE^+), which was specific for the appropriate peptide. By contrast, only 12% of the cells were PE^+ when stained at 4°C, and these exhibited ~2-fold reduced intensity. Both the minor PE^+ population detected at 4°C and the major population detected at 37°C were oligoclonal, as shown by TCR Vβ3 and Cβ1 analysis (not shown), and both had characteristics of $CD4^+$ memory T cells ($CD3^+$, $CD4^+$, $CD8^-$, $CD25^+$, $CD45RO^+$ and $CD62L^-$). These results show that most of the polyclonal T cells share with HA.17 and Cl-1 the requirement for an active cellular process to observe MHC oligomer staining, although some cells can stain in the absence of such processes.

II. C.5 Staining is reduced by treatments that induce a non-responsive state.

T cells can enter a non-responsive or anergic state in response to a partial activation stimulus (Schwartz et al, 1996). We tested whether such treatments would effect T cell staining by MHC oligomers. Treatment with high concentrations of antigenic but not control peptides (Lamb et al, 1983), or with immobilized anti-CD3 (Jenkins et al, 1990), each dramatically reduced oligomer staining of the polyclonal T cell line (Fig. II.5*b*). TCR surface expression was reduced somewhat by these treatments (Fig. II.4*c*), but the effect was much smaller and accounted for less than 10% of the overall reduction in oligomer staining (Fig. II.5*b*). These results show that MHC oligomer staining of T cells is dependent on the T cell activation state.

II. D. Discussion

The results presented here show that bound HLA-DR1 oligomers are internalized efficiently by two antigen-specific T clones. Oligomer staining was blocked by low temperature, and by endocytosis inhibitors, suggesting that an active process requiring cytoskeletal rearrangement was required. Essentially all of the fluorescence of oligomer-stained T cells resulted from internalized oligomers, and cell-surface staining was not detected. For a polyclonal $CD4^+$ T cell line, most cells exhibited the same behavior as the T cell clones, with staining blocked at 4°C. (A sub-population of $CD4^+$ T cells capable of staining at 4°C was present, consistent with the observations of other investigators, W. Kwok and G. Nepom, personal communication). Staining was greatly reduced by treatments that anergize or inactivate T cells. These results indicate that class II MHC oligomer staining of $CD4^+$ T cells require active T-cell processes, and depends on the T cell activation state.

This behavior is not likely to be limited to the particular MHC-TCR interaction investigated here. Although a detailed kinetic analysis has not yet been reported for the MHC-

TCR interactions investigated in this study (HLA-DR1, Ha peptide, and HA1.7 TCR), binding competition analysis suggests a monomeric $K_d \sim 10^{-6}$ M (Cochran & Stern 2000), within the range observed for other MHC-TCR interactions ($10^{-4} – 10^{-7}$) (Crawford et al 1998; Davis et al 1998). In addition, a temperature dependence of staining similar to that observed here has been reported previously for murine class II MHC oligomers (Crawford et al 1998). Finally, similar behavior was observed in a short-term T cell line as well as two antigen-specific T cell clones. Thus, a requirement for active cellular processes resulting in oligomer internalization may be a general (although not universal) characteristic of oligomer staining in the class II MHC system.

The requirement for active processes and association with internalization can be understood in terms of the effects of multivalent engagement of TCR. The MHC-TCR interaction is relatively weak, and would be expected to require multivalent engagement to survive the washing steps required for flow cytometry. Indeed, such considerations led to the initial development of MHC tetramers as staining reagents (Altman et al 1996). Multivalent engagement is likely to require reorganization or rearrangement of TCR molecules in the plane of the membrane (Cochran & Stern 2000). Such rearrangements are likely to require cytoskeletal participation (Kupfer & Singer 1988), and could be altered in non-responsive or anergic T cells (Viola et al, 1999). For $CD4^+$ T cells, multivalent engagement will trigger activation processes (Boniface et al 1998; Cochran et al 2000; Germain & Stefanova 1999), leading to downregulation (internalization) of activated TCR (Germain & Stefanova 1999; Valitutti et al 1995), and internalization of bound MHC oligomers.

Staining of $CD8^+$ T cells by class I MHC tetramers in general does not appear to require receptor internalization or other active T cell processes, as evidenced by their ability to be stained at 4°C (Altman et al 1996; Callan et al 1998; Yee et al 1999) (although internalization

has been reported to increase staining intensity and specificity (Whelan et al 1999)). The reason for this difference between the behavior of class I and class II MHC oligomers is not clear. It may reflect differing roles for the coreceptors CD8 and CD4 in oligomer binding or cellular activation, or an intrinsic difference in the ability to crosslink or aggregate TCRs in $CD8^+$ vs. $CD4^+$ T cells.

One of the primary attractions of using MHC tetramers to detect antigen-specific T cells has been their ability to bind T cells independent of cellular response or activation state. This has been observed for class I MHC tetramers in several studies (Altman et al 1996; Murali-Krishna et al 1998; Tan et al 1999). If our observations of the activation-dependence of class II MHC oligomer staining are a common feature of the system, they may preclude the use of class II MHC oligomers to characterize naïve, inactive or anergized $CD4^+$ T cells. These considerations may be relevant to recent reports using class II MHC oligomers to investigate responding frequencies for $CD4^+$ T cells (Kotzin et al 2000; Kwok et al 2000; Savage et al 1999).

II. E. Acknowledgments

I thank Jennifer Cochran for input and experimental assistance, Lucy Wedderburn, Abbie Meyer, and Betsy Mellins for advice regarding T-cell staining and cloning protocols, Jim Bear for help with fluorescence microscopy, Jodi Lubetsky for DR mutagenesis, Jonathan Lamb and Alessandro Sette for T-cell clones, Yassine-Diab Bader for HA03 polyclonal T cell line, and Glenn Paradis and the MIT Cancer Center Flow Cytometry facility for expert advice and assistance.

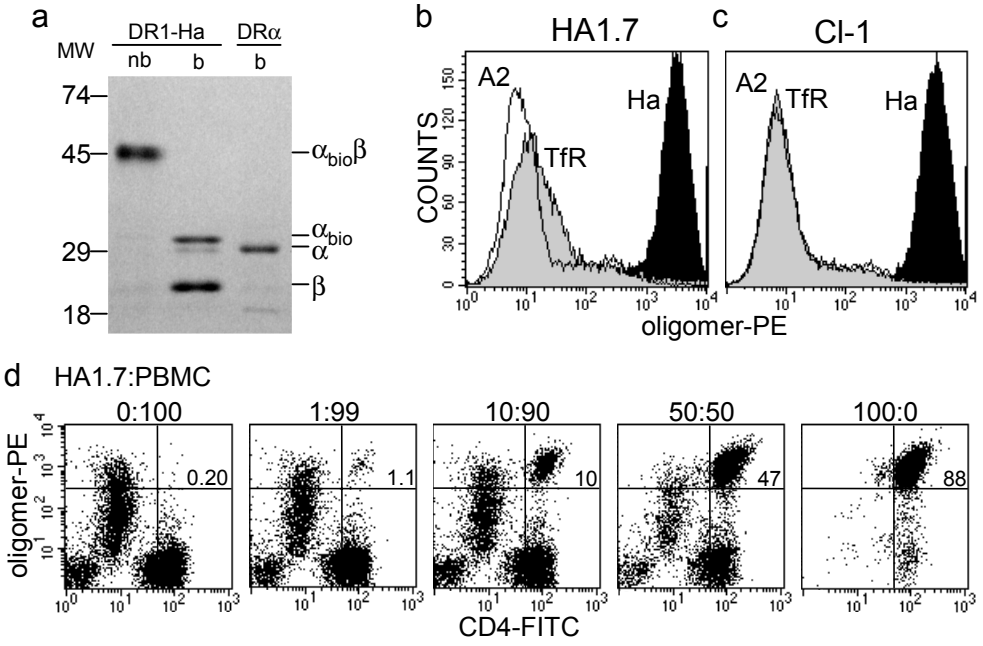

Figure II.1. *MHC class II oligomers specifically stain T cells.* (*a*) SDS-PAGE of biotinylated DR1-Ha complexes used in construction of oligomers. Heat- and SDS-resistance of αβ band (not boiled, nb) indicates formation of DR1-Ha peptide complex. Decreased mobility of DRα in the complex (middle) relative to the free subunit (right) reflects quantitative biotinylation. Flow cytometry of HA1.7 (*b*) or Cl-1 (*c*) T cells stained with SA-PE oligomers carrying DR1-Ha (solid profile), DR1-TfR (shaded profile), or DR1-A2 (open profile) peptide complexes. (*d*) Detection of HA1.7 T cells diluted into non-specific PBMC mixtures at indicated ratios, and stained with FITC-CD4 and DR1-SA-PE oligomer carrying Ha peptide. Percentage of DR1-SA-PE$^+$ CD4$^+$ cells in each mixture is indicated in the upper-right hand quadrant.

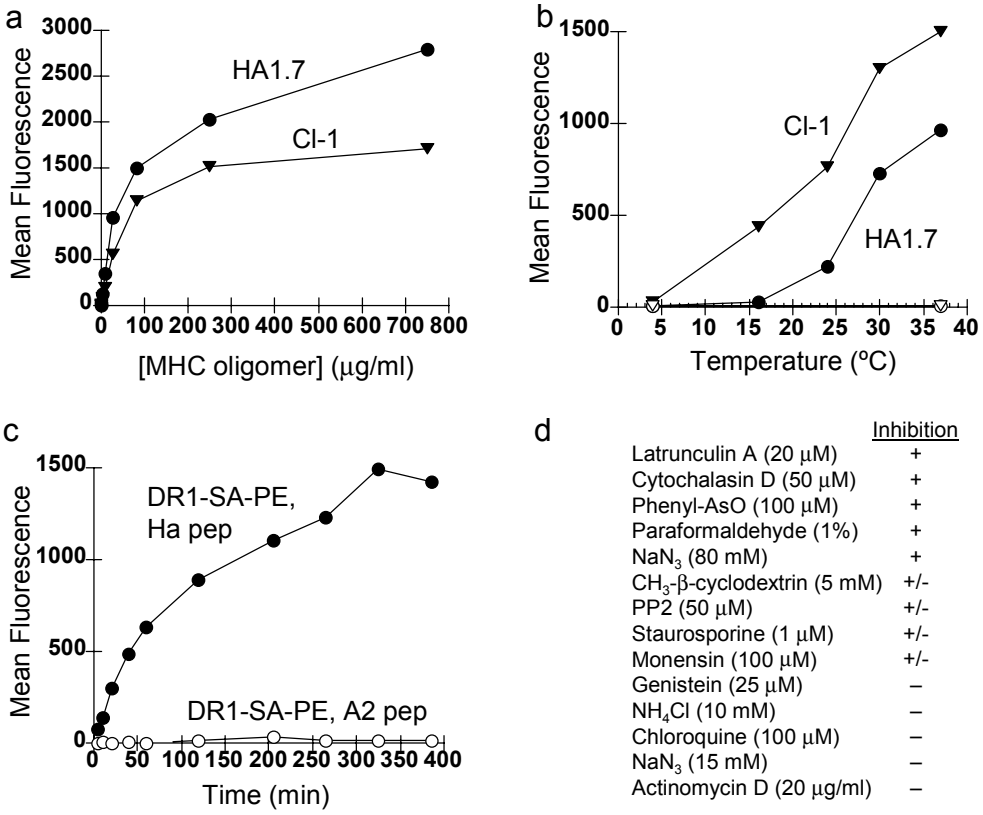

Figure II.2. *MHC oligomer staining depends on experimental conditions.* (*a*) Concentration, (*b*) temperature and (*c*) time dependence of staining for HA1.7 (circles) and Cl-1 (triangles) cells. Standard conditions (50 μg/ml, 37°C, 5 hours) were varied as noted. (*d*) Inhibitor analysis. Fraction of signal observed in the treated relative to an untreated sample is shown (+ <40%; +/- 40-70%; – >90%).

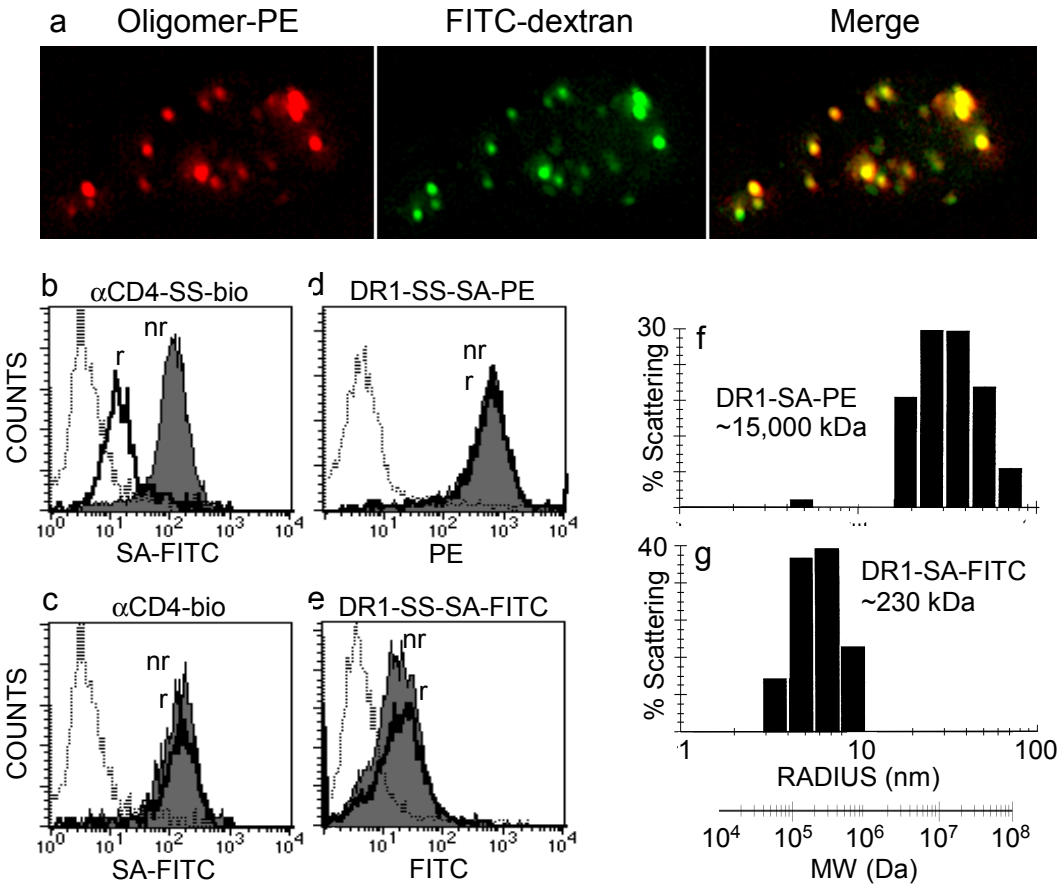

Figure II.3. *Cell-associated oligomers are not present at the surface.* (*a*) Fluorescence microscopy. Three-dimensional reconstruction of a single HA1.7 cell after incubation with DR1-Ha SA-PE oligomer (red) and the fluid-phase endocytosis marker FITC-dextran (green). (*b,c,d,e*) Surface stripping. Oligomer- or antibody-stained HA1.7 T cells (nr, not reduced, shaded trace) were stripped with the membrane-impermeant reducing agent βME-SO_3^- (r, reduced, open trace). Control staining (dotted traces) is shown for unstained cells (*b,c*) or for cells stained with oligomers carrying non-antigenic TT peptide (TT, *d,e*). βME-SO_3^- strips antibody (αCD4-SS-bio) but not MHC oligomer (DR1-SS-SA-PE, DR1-SS-SA-FITC), or non-cleavable antibody control (αCD4-bio). (*f,g*) Dynamic light scattering. Hydrodynamic radii distributions were estimated by dynamic light scattering for (*f*) DR1-SA-PE oligomer (average=34.4 nm), and (*g*) DR1-SS-SA-FITC tetramer (average=6.1 nm). Corresponding molecular weights are 15,000 kDa and 230 kDa, estimated using a model for globular proteins.

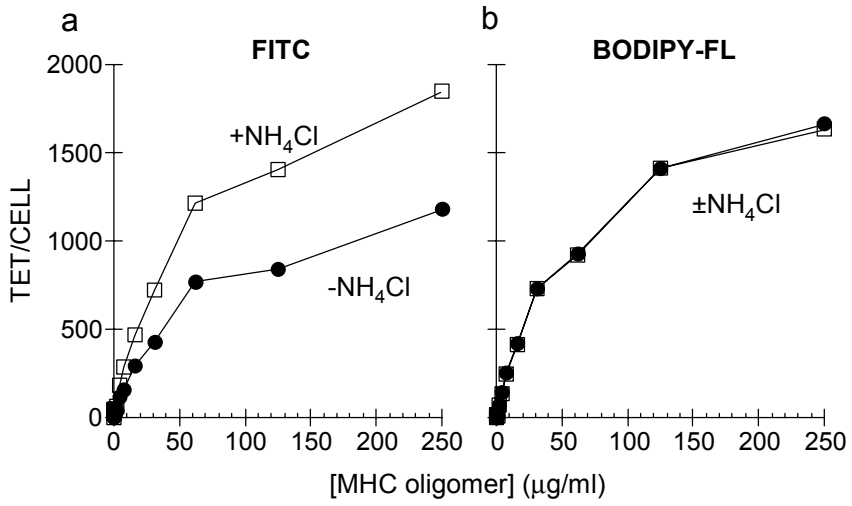

Figure II.4. *Cell-associated class II MHC tetramers are found in acidic vesicles.* HA1.7 cells were stained with (*a*) DR1-Ha SA-FITC (pH-sensitive) or (*b*) DR1-Ha SA-Bodipy-FL (pH-insensitive) tetramer, and analyzed by flow cytometry before (filled circles) or after (open squares) intracellular neutralization by addition of NH_4Cl.

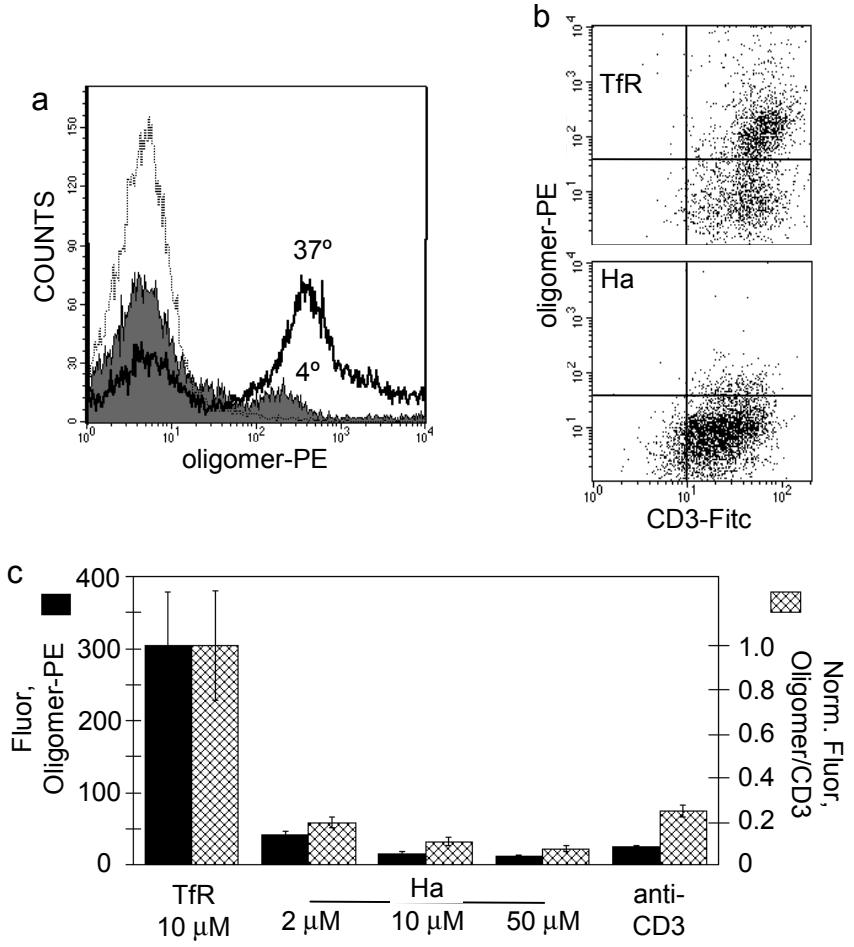

Figure II.5. *Staining of a polyclonal T cell line.* (*a*) Cells were incubated with DR1-SA-PE carrying Ha peptide at either 37°C (open trace) or 4°C (shaded trace) for 3 hours before analysis by flow cytometry. Staining with control oligomer is shown as dashed trace. When stained at 37°C, 67% of the cells were PE^+, whereas when stained at 4°C only 12% were PE^+. (*b*) Treatments that anergize or inactivate T cells block staining. Cells were incubated overnight with the indicated concentration of control (TfR) or specific (Ha) peptide, or with immobilized anti-CD3 antibody, before DR1-SA-PE staining. Closed bars, oligomer staining intensity; hatched bars, staining after normalization for CD3 expression. (*c*) MHC oligomer and CD3 staining levels after pre-treatment with 10 μM control (TfR) or specific (Ha) peptide.

III. Labeling antigen-specific $CD4^+$ T cells with class II MHC oligomers

Here we review methodologies for production of fluorescent oligomers of soluble class II MHC proteins and discuss their use in analysis of antigen-specific $CD4^+$ T cells. Class I MHC-peptide oligomers (MHC tetramers) have become popular reagents for the detection and characterization of antigen-specific $CD8^+$ T cells. Class II MHC proteins can be produced by expression in *E. coli* followed by *in vitro* folding, or by native expression in insect cells; biotin can be introduced by site-specific chemical modification of cysteine, or by enzymatic modification of a peptide tag; and a variety of fluorescent streptavidin preparations can be used for oligomerization. We explore the experimental conditions necessary for efficient staining of $CD4^+$ T cells using oligomers of class II MHC proteins, and we establish a standard protocol. Finally we consider complications and challenges associated with these reagents, discuss the interpretation of staining results, and suggest future directions for investigation, in particular the use of MHC oligomers for the study of T cell avidity modulation

III.A. Introduction

In order to understand and manipulate the immune response it is critical to identify and characterize the responses to particular antigens. The analysis and detection of specific antibodies is well developed, and assay of antigen-specific antibody responses is now routine. However, investigation of the other branch of the adaptive immune response, antigen-specific T cells and their clonotypic T cell receptors, has been much more difficult. Cellular methods are available that identify the capability of specific T cells to proliferate in response to antigen, to secrete cytokines, or to kill target cells, and there are molecular techniques to characterize the TCR sequence diversity (or lack thereof) in a responding population. But none of these

methods can directly identify antigen-specific T cells, independent of their functional capacity. This is critical, as phenomena that might obscure or blunt T cell functions, including T cell tolerance, exhaustion, suppression, and anergy, have become increasingly important in immunological research. The technique of MHC tetramer staining of T cells, developed by Altman and colleagues (Altman et al 1996), has enabled the direct identification of antigen-specific $CD8^+$ T cells in principle based on TCR specificity and not T cell function or TCR sequence.

Recently, this MHC-tetramer technology has been extended to the use of class II MHC tetramers to stain $CD4^+$ T cell populations (Cameron et al 2001; Crawford et al 1998; Kwok et al 2000; Meyer et al 2000; Novak et al 1999; Savage et al 1999). Some of these results indicate that the application of MHC tetramer technology to $CD4^+$ T cells may not be straightforward. In particular, $CD4^+$ T cell staining appears to be sensitive to the MHC-TCR affinity (Crawford et al 1998) and the activation state of the T cell (Cameron et al 2001), and responding populations of human $CD4^+$ T cells often are too small to be analyzed without amplification *in vitro* (Kwok et al 2000). These barriers to the routine use MHC tetramer staining, while present to some extent in the analysis of $CD8^+$ T cells, appear to be more formidable in the analysis of $CD4^+$ T cells.

Here, we compare the methods that have been described for production of class II MHC-peptide complexes and their biotin derivatives, we evaluate various fluorescent streptavidin reagents used to prepare MHC oligomers, and we demonstrate the use of HLA-DR1 oligomers in staining $CD4^+$ T cell clones, polyclonal lines, and samples of peripheral blood. We describe the varying temperature requirements for staining $CD4^+$ T cells, and we

discuss the relation between oligomer staining, T cell specificity and cellular function. Finally, we consider the future of these reagents in molecular and clinical immunology.

III.B. Materials and Methods

III.B.1 *Peptides*

Peptides Ha[306-318] (PKYVKQNTLKLAT), FluB[308-320] (PYYTGEHAKAIGN), p24(34)[34-46] (PEVIPMFSALSEG), A2[103-114] (VGSDWRFLRGYHQYA) and TfR [680-696] (RVEYHFLSPYVSPKESP) were synthesized using solid-phase Fmoc chemistry, purified by C18 reverse-phase HPLC, and verified by MALDI-TOF mass spectrometry. Ha is an antigenic peptide from A-strain influenza hemagglutinin (Lamb et al 1982), FluB is an antigenic peptide from B-strain influenza hemagglutinin (Robbins et al 1997), p24(34) is an antigenic peptide derived from HIV-1 p24 (Norris et al 2001), and A2 and TfR are control peptides originally identified as highly abundant peptides in MHC proteins present in a B cell line (Chicz et al 1992).

III.B.2 *Antibodies and Streptavidin*

Mouse monoclonal antibody OKT4 (anti-human CD4) (ATCC, Manassas, VA) was purified from hybridoma supernatant by protein-A Sepharose (Repligen Corp, Needham, MA). For fluorescent labeling, antibody or streptavidin (Prozyme Inc, San Leandro, CA) was incubated with ten-fold molar excess FITC (Sigma-Aldrich, St. Louis, MO) in 10% DMSO, or Alexa-488 succinimide ester (Molecular Probes, Eugene, Oregon) in aqueous solution, for 3 hours at room temperature, followed by gel filtration using Sephadex G-50 (Pharmacia, Piscataway, NJ). We previously reported a technique for protecting biotin-binding sites with 2-

hydroxyazobenzen-4'-carboxylic acid (Haba, Pierce Chemical Co.) during fluorescent labeling (Cameron et al 2001). However, after careful optimization of labeling conditions, we find that this step is unnecessary for the production of highly-labeled, highly-active SA. R-phycoerythrin conjugated streptavidin (SA-PE) was purchased from Biosource, Inc, Camarillo, CA. Each lot of SA-PE was individually titrated with DR1-peptide and used to stain the HLA-DR1-restricted, HA-peptide-specific, long term T_H0 T cell clone HA1.7 (Lamb et al 1982) to determine the SA-PE:DR1 ratio that provided maximal staining. Allophycocyanin-conjugated anti-human CD4 antibody was purchased from Diatec AS, Norway.

III.B.3 *DR subunit bacterial expression*

Soluble HLA-DR1 was produced by expression of individual subunits in *E. coli*, followed by folding *in vitro* according to a previously reported protocol (Frayser et al 1999). Truncated HLA-DR1 alpha (DRA*0101) and beta (DRB1*0101) genes missing transmembrane and cytoplasmic domains (α1-190, β1-192) were each cloned behind a T7 promoter in the pLMI vector (MacFerrin et al 1990) after modification of the alpha chain to carry either a C-terminal Ala-Cys sequence ($DR\alpha_{cys}$) (Cochran & Stern 2000) or C-terminal biotin-ligase substrate peptide tag (Schatz 1993) (GSLHHILDAQKMVWNHR) ($DR\alpha_{BSP}$, a generous gift of Souheil Younes and Rafick Pierre Sekaly, Univ. of Montreal). BL21 DE3 *E. coli* were transformed with either pLMI DRα or pLMI DRβ plasmids and stored as glycerol stocks at –70°C. Overnight cultures were used to seed 1-10 liters of Luria Broth supplemented with 50 μg/ml ampicillin and 0.2% D-glucose, induced at OD_{600} 1.0-1.5 with 0.5 mM IPTG, and harvested three to five hours later.

III.B.4 *Isolation of crude inclusion bodies*

Inclusion bodies containing DR subunits were isolated using a modified detergent extraction protocol (Nagai & Thogersen 1987). *E. coli* cell pellets from ten liter culture were resuspended in 200 ml sucrose solution (50 mM Tris-Cl pH 8.0, 25% sucrose, 1 mM EDTA, 0.1% Na azide, 10 mM DTT, freshly dissolved), 100 mg lysozyme were added, the solution was stirred at room temperature for ten minutes, and cells were lysed by the addition of 500 ml of deoxycholate/triton solution (20 mM Tris-Cl pH 8.0, 1% Na Deoxycholate, 1% Triton X-100, 100 mM NaCl, 0.1% Na azide, 7 mM $MgCl_2$, 10 mM DTT). One mg DNase I was added, the solution was stirred until its viscosity was reduced significantly (10-20 minutes), and then frozen at -20ºC. Solutions were later thawed, centrifuged at 6000xg, and the pellet of inclusion bodies was resuspended in 200 ml triton solution (50 mM Tris-Cl pH 8.0, 0.5% Triton X-100, 100 mM NaCl, 1 mM EDTA, 0.1% Na azide, 1 mM DTT) using a polytron homogenizer (Brinkmann Inst., Westbury, NY). The inclusion bodies were centrifuged and resuspended three more times in triton solution, and twice more in tris solution (50 mM Tris-Cl pH 8.0, 1 mM EDTA, 0.1% Na azide, 1 mM DTT). Pellets were finally solubilized in 50-200 ml urea solution (8 M urea, deionized over mixed-bed ion-exchange beads (Sigma-Aldrich), 20 mM Tris-Cl pH 8.0, 0.5 mM EDTA, 0.1% Na azide, 10 mM DTT) and immediately frozen at –70ºC.

III.B.5 *Ion exchange purification of DR inclusion bodies*

Crude inclusion body pellets were thawed, centrifuged at 6,000xg to remove insoluble material, filtered through 0.45 μm membranes, and treated with additional 30-50 mM DTT for 10-20 minutes at room temperature to ensure complete reduction of cysteines. On a

Perseptives Sprint HPLC (Applied Biosystems, Foster City, CA), a 20-30 ml column of Poros HQ20 resin (Applied Biosystems) was equilibrated with 8 M deionized urea, 20 mM Tris-Cl pH 8.0 (for α) or 9.0 (for β), and loaded with approximately 150 mg of crude inclusion bodies at the same pH. Protein was eluted by a 0-500 mM NaCl gradient over 10 column volumes. Eluted protein was collected as three to five different fractions, small aliquots were saved for analysis by SDS-PAGE and test folding reactions, and the remainder was immediately frozen at –70ºC.

III.B.6 *In vitro folding, and purification of DR1-peptide complexes*

Folding buffer (20 mM Tris-Cl pH 8.5, 0.5 mM EDTA, 1 mM GSH, 0.1 mM GSSG, 25% glycerol) containing 0.4 μM peptide was chilled and purified inclusion bodies were added dropwise to the stirring buffer to a final concentration of 10 mg per liter of each subunit. Reactions were incubated at 4°C for two days.

MHC-peptide complexes were collected by the addition of 2.5 g dry DEAE sephadex A-50 (Sigma-Aldrich) per liter folding mix (no stirring), allowed to swell overnight, and mixed by inversion several times the following morning. DEAE-sephadex was collected on Whatman filter paper in a Buchner funnel, and washed with 20 mM Tris-Cl pH 8.0, 1 mM DTT. To elute the collected protein, 5 M NaCl (3.5 ml per dry gram of DEAE resin) was added and immediately stirred into the caked beads in the funnel. After ten minutes, eluate was collected and filtered again to eliminate any DEAE beads that might have been carried over. The filtrate was dialyzed in 10,000 MW cutoff membranes (Spectrum Labs, Rancho Dominguez, CA) versus 10 volumes of 10 mM Tris-Cl pH 8.0, 20 mM NaCl, 1 mM DTT for 3-5 hours at 4°C. Alternatively, the filtrate can be exchanged into the same buffer by tangential ultrafiltration

using a Pellicon 10k cartridge (Millipore, Bedford, MA). The buffer-exchanged sample was centrifuged at 6,000xg to remove insoluble material, and filtered through 0.45 μm membranes.

DR1-peptide complexes were purified from contaminating misfolded or empty DR complexes by native anion exchange chromatography. The buffer-exchanged filtrate was treated with 20 mM freshly-dissolved DTT at room temperature for 20 minutes to ensure reduction of the C-terminal cysteine (the intrasubunit disulfide bonds in the native protein are resistant to reduction under these conditions), diluted with 20 mM Tris-Cl pH 8.0 solution to a final salt concentration between 20 and 50 mM (confirmed by conductivity), and loaded onto a 1-3 ml Poros HQ 20 column pre-equilibrated in 20 mM Tris-Cl pH 8.0. Folded DR1-peptide$_{cys}$ was eluted by a gradient into 20 mM Tris-Cl pH 8.0, 0.5 M NaCl over 20 column volumes. DR1-peptide complexes typically eluted at approximately 120 mM NaCl. Overall yield of folded DR1-peptide complexes was typically 0.05-0.3 mg per liter of folding mix.

III.B.7 *Chemical biotinylation of DR1$_{cys}$*

For biotinylation of DR1$_{cys}$ constructs, DR-containing fractions were pooled, freshly dissolved biotinyl-3-maleimidopropionabmidly-3,6-dioxaoctanediamine (PEO-maleimide Biotin, Pierce Chemical Co.) was added to a final concentration of 50 μM, and the mixture was incubated for 10-30 minutes at room temperature. Excess reagent was quenched by the addition of 1 mM DTT. Protein was concentrated in a 10,000 MW cutoff spin ultrafiltration device (Centricon-10, Millipore, Bedford, MA) and purified on a gel filtration column (SEC-3000, Phenomenex, Torrance, CA) in phosphate buffered saline (PBS, 15 mM Na/K PO_4, 135 mM NaCl, pH 7.0) to remove excess biotin. Alternatively, excess biotin can be removed by extensive dialysis or repeated cycles of concentration and dilution in spin ultrafiltration

devices. Biotinylated protein was concentrated in a Centricon-10 to a final concentration 1-5 mg/ml. Chemically biotinylated DR1-peptide complexes were observed to be stable for at least twelve months when stored at 4ºC.

III.B.8 *Enzymatic biotinylation of $DR1_{BSP}$*

As an alternative to chemical biotinylation, a C-terminal BSP tag can be enzymatically biotinylated using biotin-ligase (Avidity, Denver, CO)(Schatz 1993). $DR1_{BSP}$ was prepared as described for $DR1_{cys}$. After HQ purification, fractions containing DR1 were pooled, concentrated in a Centricon-10 to 1 mg/ml, exchanged into reaction buffer (50 mM Bicine pH 8.3, 10 mM ATP, 10 mM MgOAc, 50 μM biotin), supplemented with 5 μg/ml biotin ligase (Avidity, Denver, CO), and incubated 30 minutes at room temperature. Excess biotin was removed by gel filtration (SEC-3000 column, PBS), by extensive dialysis, or by multiple cycles of concentration and dilution.

III.B.9 *$DR1_{cys}$ expression in insect cells*

Expression of $DR1_{cys}$ in insect cells was initially performed using baculoviral infection of Sf9 cells essentially as described (Stern & Wiley 1992). Subsequent expression using stable transfection of S2 Schnieder cells (Bunch et al 1988) was found to provide equivalent or greater protein yield with less experimental effort. Expression vector pRMHa-3 and resistance vector pNeo were gifts from K. Christopher Garcia (Stanford University). S2 Schneider cells (ATCC) and pRMHa-3 containing DRβ (1-192) were generous gifts from Elizabeth Mellins (Stanford University). DRα(1-190)-Ala-Cys (including its native signal sequence) was cloned into pRMHa-3 using standard molecular biology techniques. S2 cells were transfected with

pRMHa-3 DRα_{cys} (1 μg), pRMHa-3 DRβ (1 μg), and pNeo (0.05 μg) by calcium phosphate (Gibco Life Technologies, Rockville, MD), and a stable transfected cell line was established by selection under 1.0 mg/L geneticin (Gibco) for four weeks. Cell stocks were frozen in 10% DMSO and stored in liquid nitrogen.

Cultures were gradually adapted to Sf900 medium (Gibco) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin (Gibco), 250 μg/L amphotericin B and 2 mM L-glutamine (Gibco). Six liter cultures were grown in stirred vessels (Bellco Glass, Vineland, NJ) at 22-24°C while being bubbled gently with filtered air. Cells were induced at a density of 5-10 x10^6 per ml by addition of 0.5 mM $CuSO_4$, and culture supernatant was collected four to six days later by centrifugation at 4000xg. Supernatant was concentrated 10-fold in a 10,000 molecular weight cutoff spiral filtration device (Millipore). DR1$_{cys}$ was purified by immunoaffinity with a LB3.1-conjugated protein A column as described (Frayser et al 1999). Protein was eluted with 50 mM CAPS, pH 11.3, and pH was adjusted to approximately 8 using 1 M Tris-HCl.

For biotinylation by chemical modification, maleimide biotin was added to the immunoaffinity fractions immediately after neutralization to a final concentration of 50 μM, and the mixtures were reacted at room temperature for 20-30 minutes and quenched with 1 mM DTT. Protein was exchanged into PBS, pH 7.2 and concentrated in a Centricon-10 (Millipore) to 1-5 mg/ml. Peptides were loaded into the purified MHC proteins by extended incubation (2-3 days) at 37ºC in the presence of an appropriate concentration of peptide (usually 3-5 fold molar excess) in solution containing 1 mM EDTA, 0.01% sodium azide, and 1 mM PMSF. HLA-DR proteins isolated from S2 cells sometimes appeared to carry heterogeneous mixtures of weakly bound endogenous peptides, as judged by non-denaturing SDS-PAGE; these

peptides could be exchanged efficiently for added peptide. DR1-peptide-biotin complexes were isolated by gel filtration chromatography (SEC-3000) in PBS and concentrated to 1-5 mg/ml for storage at 4ºC. Final yield was approximately 0.1-0.3 mg DR1-peptide-biotin per liter S2 cell culture.

II.B.10 *Oligomerization*

For oligomerization using SA-PE, an optimal ratio of DR to SA-PE was determined for each lot of SA-PE by staining HA1.7 T cells. SA-PE was added stepwise to 0.1-5 mg/ml biotinylated DR1-peptide complexes in PBS to the empirically determined final ratio, usually in four steps with approximately 1 minute between additions. For oligomerization using SA-FITC or SA-Alexa, the SA-fluorophore was added in four steps to a final molar ratio of one SA to four DR. SA-FITC and SA-Alexa protein concentrations and fluor/protein ratios were determined by UV-Vis spectrophotometry utilizing ε_{280} values of 18,800 M^{-1} for SA, 15,200 M^{-1} for FITC, 8,000 M^{-1} for Alexa-488, and ε_{490} values of 69,000 M^{-1} for FITC and 78,000 M^{-1} for Alexa-488.

SA-FITC and SA-Alexa oligomers prepared at various SA:MHC ratios were analyzed by gel filtration. For these experiments, SA-fluorophore was added to DR1 in a single step. High-resolution gel filtration was accomplished by using two columns in series (tandem gel filtration). The experiments described here were performed with an upstream SEC-3000 column (Phenomenex) and downstream Sephadex 200 column (Pharmacia), in PBS at 0.5 ml/min for 100 min. We have found other combinations of high-performance gel filtration columns to be equally effective.

For characterization of the size of DR1 oligomers formed with either SA-PE or SA-FITC, dynamic light scattering measurements were made using a Protein Solutions DynaPro-MS/X instrument thermostatically controlled at 22°C. Protein samples were filtered through 0.2 micron spin filters (Costar) before analysis. Molecular weight equivalents (Da) were calculated from hydrodynamic radii (nm) using a standard curve model for globular proteins, $MW = (1.549 x R_H)^{2.426}$.

II.B.11 *T cell clones and lines*

T cell clones HA1.7 (Lamb et al 1982), Cl-1 (De Magistris et al 1992), HaCOH8 were cultured in RPMI with 5% human serum and 5% fetal bovine serum, 50 units/ml penicillin G (Gibco), and 50 μg/ml streptomycin sulfate (Gibco). These lines are all specific for the Ha peptide bound to HLA-DR1. They were maintained by stimulation every 2-3 weeks with an irradiated mixture of non-autologous peripheral blood lymphocytes and a DRB1*0101 EBV-transformed B cell line (EBV1.24) that had been pulsed with 1 μM Ha peptide. Every 3-4 days, 40 units/ml interleukin-2 (Aldesleukin, Chiron Corp., Emeryville, CA) was added to the cultures.

Short-term polyclonal T cell lines were raised by *in vitro* stimulation of lymphocytes isolated from $DR1^+$ volunteers. Freshly isolated PBMCs were first labeled with 1 μM CFSE (Molecular Probes) by incubation for 10 min. at 37°C in RPMI, and then quenched with three washes in media. PBMCs were then aliquoted into 24-well plates at 5 million lymphocytes per well, and stimulated by the addition of 5-20 μM peptide. On days 7 and 10 after stimulation, 40 units/ml IL-2 was added. Secondary stimulation on day 14 with 5 μM peptide and non-

autologous PBMCs and $DR1^+$ EBV-transformed B cells was performed as described as above, followed by IL-2 addition every 3-4 days.

The DR1-Ha specific clone HaCOH8 was derived as previously described (Moulon et al 1998). Briefly, $DR1^+$ PBMCs were stimulated with 7 μM Ha peptide in media, and supplemented on day 7 with IL-2 (100 units/ml). On day 12, were stimulated with irradiated autologous PBMC and Ha, and after 3 days supplemented again with IL-2. For cloning, T cell blasts were seeded at 0.3 cells per well in terasaki plates (Nalgenunc Int., Rochester, NY) in the presence of 1 μg/ml PHA-P (Sigma-Aldrich), 100 units/ml IL2 and irradiated fresh allogeneic PBMC. The obtained T cell clones were expanded and maintained in culture by periodic stimulation in the presence of irradiated allogeneic PBMC, PHA and IL-2.

The HIV-1 p24-specific clone from $DR1^+$ subject AC-25 was derived via limiting dilution. Freshly isolated PBMC ($1x10^7$) were suspended in 10ml of media in a T25 flask and stimulated with p24 (1 μg/ml, Protein Sciences, Meriden, CT) and IL-2 (100 units/ml, Hoffman La Roche). For the first four weeks of culture, the media was supplemented with Indinavir (Merck, 0.4 μM), AZT (Glaxo Wellcome, 0.5 μM), and 3TC (Glaxo Wellcome, 3 μM) to block HIV replication. After *2* weeks the PBMC were restimulated with p24 protein (1 μg/ml), IL-2 (100 units/ml), and 10^7 irradiated, autologous PBMC. Three days later PBMC were plated at limiting dilution. A clone from AC-25 was found to p24-specific and was maintained with restimulation every 2 weeks with the anti-CD3 antibody 12F6 (obtained from Dr. Johnson Wong, Massachusetts General Hospital), IL-2, and 10^7 irradiated allogeneic PBMC. All samples of human peripheral blood were taken from volunteers after the nature of the study and

possible consequences of participation had been fully explained and informed consent had been obtained.

A Jurkat T cell mutant selected for lack of TCR expression and subsequently transfected with genes coding for the HA1.7 TCR, called CH7C17 (Hewitt et al 1992; Wedderburn et al 1995), was passaged in RPMI supplemented with 10% fetal bovine serum, penicillin G, streptomycin, L-glutamine, 400 μg/ml hygromycin, 4 μg/ml puromycin. RBL mast cells transfected with HA1.7 TCR/zeta chimeric constructs, Y22.D6 (Seth et al 1994), were passaged in DMEM, 20 mM Hepes pH 7, 15% FBS, penicillin, streptomycin, L-glutamine, 1 mg/ml G418.

II.B.12 *Flow cytometric staining of T cells*

Oligomer staining of T cells was detected by flow cytometry. In order to conserve reagent, staining reactions were performed in the smallest practical volumes. Typically, equal volumes of DR1-peptide oligomer reagent in RPMI and T cells in culture medium were mixed to a final concentration of 20-50 μg/ml oligomer, in 10-20 μl of culture medium in round-bottom or v-bottom 96-well plates, and sealed with packing tape. For staining at 4°C, plates, cells and oligomers were pre-chilled on ice. Stainings were performed for 3-5 hours. Higher concentrations of oligomer and longer staining times have been shown to result in brighter staining (Cameron et al 2001), but weren't utilized in the studies described herein to conserve both reagent and experimental effort. At the end of the oligomer staining reaction, samples were chilled for five minutes, and stained with CD4-APC for 20-30 minutes on ice. Samples were washed twice with cold wash buffer (PBS, 1% fetal bovine serum, 15 mM sodium azide)

and analyzed by flow cytometry (Becton Dickinson FACScan or FACScaliber) as quickly as possible.

III.C Results

III.C.1 *DR1 subunit production in E. coli*

Originally, soluble class II MHC proteins were prepared from B cell lines by proteolytic cleavage between the extracellular and transmembrane domains (Gorga et al 1987). These preparations contained heterogeneous high-affinity peptides and were difficult to load homogenously with a peptide of choice. To produce homogenously loaded MHC-peptide complexes, two popular methods have been developed subsequently: expression of MHC subunits in *E. coli* followed by *in vitro* folding in the presence of peptide (Fig. III.1, left panel), and secretion of soluble folded MHC protein from insect cells followed by loading of empty molecules and/or displacement of weakly-bound insect-cell peptides using high concentrations of exogenous peptide (Fig. III.1, right panel).

For HLA-DR1, our preferred method for production of soluble MHC-peptide complexes is to fold them *in vitro* using denatured inclusion bodies purified from *E. coli* as described in detail previously (Frayser et al 1999). To enable site-specific biotinylation of MHC-peptide complexes the alpha subunit of DR1 was modified either with a C-terminal Ala-Cys dipeptide for chemical biotinylation, or with a C-terminal 15-residue biotin-ligase substrate peptide (BSP) sequence for enzymatic biotinylation. DR1 subunit constructions were cloned into T7-promotor vectors, and DRα_{cys}, DRα_{BSP}, and DRβ subunits were expressed in *E. coli* using methods developed for the unmodified protein (Frayser et al 1999). Figure III.2a shows SDS-PAGE analysis of total cell lysates before and after induction of DR subunits (NI and I,

respectively). The induced α and β subunits are observed at their expected molecular weights and represent 19% and 13%, respectively, of total *E. coli* protein, as analyzed by densitometry. After washing the insoluble inclusion bodies repeatedly with detergent solution, the subunits were solubilized in urea (IB) and subsequently purified by anion-exchange chromatography (HQ). *In vitro* folding is accomplished by rapid dilution of the denatured DR1 α and β subunits into a large volume of pH- and redox-buffered solution containing peptide of choice, extended incubation at low temperature with folded protein recovered by anion exchange chromatography several days later. Misfolded and/or empty DR1 is lost during the purification procedures. DR1-peptide complexes generally are stable to SDS-induced chain dissociation at room temperature (Stern & Wiley 1992). The folded DR-peptide complexes in Figure III.2b migrate as intact complexes (- boil). The absence of free α or β subunits from the non-boiled samples indicates quantitative peptide loading in the purified protein (compare + and – boil lanes).

DR1-peptide complexes were biotinylated either by chemical reaction of a carboxy-terminal cysteine with a maleimide-biotin reagent, or by enzymatic ligation of biotin to the carboxy-terminal biotin-ligase substrate peptide (BSP) (Fig. III.3). Biotinylation of $DR\alpha_{cys}$ can be observed by a slight shift in the mobility of the subunit in gel electrophoresis (Fig. III.2b, compare $DR\alpha_{cys}$ to the $DR\alpha_{bio}$ band of boiled DR-pep_{cys} complexes). However, biotinylation is more clearly demonstrated by the supershift of $DR\alpha_{bio}$ after the addition of excess SA (+ SA lanes). This analysis indicated that both chemical reaction and enzymatic ligation result in high efficiency biotinylation (>90%). In our experience, both chemical and enzymatic biotinylation are effective, stable, and facile. The thiol modification strategy has been extended to produce oligomeric forms and topologies not available using streptavidin-mediated coupling (Cochran

et al 2000). The full variety of strategies used to prepare MHC oligomers and chimeras includes several that do not utilize streptavidin, and has been reviewed separately (Cochran et al 2001).

III.C.2 *Production of soluble DR1-peptide complexes from insect cells*

As an alternative to *E. coli*, insect cells can be induced to secrete correctly folded and assembled DR1 (Stern & Wiley 1992). DR1 expressed by this method is isolated from the culture medium by immunoaffinity chromatography, loaded with peptide of choice, and biotinylated, as shown schematically in Fig. III.1, right panel. Two systems have been described for MHC production in insect cells. In the initial studies, MHC proteins were produced in Sf9 *Spodoptera fr.* cells by infection with recombinant baculovirus carrying the MHC genes, either separately or on a dual-promoter virus (Kozono et al 1994; Stern & Wiley 1992). However, the labor-intensive and highly variable virus production step required in the baculovirus system has led many researchers to use a system of stable transfection in S2 Drosophila cells (Sloan et al 1995). DR1 produced in either of these systems has behaved similarly in our hands. DR1-pep_{cys} produced in S2 cells was >70% biotinylated by chemical modification (data not shown). Although not investigated in our laboratory, enzymatic biotinylation of insect cell derived MHC has been reported at high efficiencies (Crawford et al 1998). In this paper we have compared DR1-peptide-biotin produced in *E. coli* and S2 cells, and we find them to be nearly identical for the purposes of oligomer staining. S2-produced protein can be quantitatively loaded with peptide of choice (Fig. III.4a), biotinylated, and used to stain T cells with comparable brightness to DR-peptide complexes produced in *E. coli* (Fig. III.4b). In general, preparations of DR1 produced in insect cells are of somewhat lower purity,

and may not be as completely loaded with the desired peptide, as DR1 produced from *E. coli* (Frayser et al 1999). However, for routine oligomer staining protocols, either preparation is suitable.

III.C.3 *Oligomerization using fluorescent streptavidin reagents*

Following the method originally described for class I MHC proteins (Altman et al 1996), we oligomerized biotinylated DR1 using either SA-FITC or SA-PE reagents. However, we observed that SA-PE oligomers of DR1 provided vastly brighter staining of $CD4^+$ T cells (compare Fig. III.5a vs. 5b). Although PE is a brighter fluorophore than FITC, the difference was greater than the relative fluorescent efficiencies intrinsic to the fluorophores, and we investigated the biochemical and physical state of the SA-FITC and SA-PE complexes.

Fluorescent modification can alter the effective valency of SA, whose maximal valency is normally four. Modification of SA with small molecule fluorophores carrying chemically-reactive linkers (such as fluorescein isothiocyanate or Alexa-488 succinimide ester) can block biotin binding sites, and so SA-FITC and SA-Alexa reagents may exhibit an effective valency less than four. Protein fluorophores, such as R-phycoerythrin (PE, 240 kDa) are difficult to conjugate to SA without causing some degree of SA:SA crosslinking. In our experience, commercially available SA-PE preparations, even those with average SA:PE ratios of 1:1, contain large crosslinked complexes of multiple SA and multiple PE, with apparent valency greater than four.

Dynamic light scattering measurements of each kind of DR1 oligomer confirmed these observations (Fig. III.5). DR1-SA-PE oligomer exhibits a hydrodynamic radius of 34 nm, which correlates with a globular protein of molecular mass greater than 15,000 kDa (Fig.

III.5a). In contrast, DR1 oligomerized with SA-FITC (Fig. III.5b) demonstrates a radius of 6.1 nm, consistent with a molecular mass of 230 kDa, close to the 260 kDa expected for a complex of one SA with four DR1. In fact, SA-PE alone exhibits a hydrodynamic radius of 32 nm, which correlates with a globular protein of molecular mass greater than 12,000 kDa (data not shown). Thus, the very bright signals observed with SA-PE appears to be due in part to SA crosslinking, in addition to the intrinsic brightness of the PE fluorophore. This point is under-appreciated by most users and suggests that these reagents would be more properly called MHC oligomers rather than MHC tetramers.

Because of the uncertainty in the actual valency of each type of SA-conjugate, conditions for optimal oligomerization should be determined for each batch of reagents. The size of SA-PE conjugates precludes analysis by gel filtration. Instead, SA-PE-based oligomerization was optimized empirically by testing various SA-PE:DR ratios for their ability to stain HA1.7 T cells (not shown). SA-Alexa or SA-FITC DR oligomers were readily analyzed by gel filtration. Figure III.6a,b shows high resolution gel filtration analyses of a titration of SA-Alexa with DR1-TfR-biotin. Uncomplexed MHC, SA, MHC-SA monomers, and most oligomeric species can be distinguished based on their elution position and absorbance characteristics. Unexpectedly high molar ratios of DR to SA-Alexa were required to obtain maximum saturation of SA with MHC molecules (approximately six DR per SA for the experiment shown in Fig. III.6) and even at saturation, some SA-DR trimers are present. These results might be explained by a combination of incomplete MHC biotinylation, sub-optimal specific activity in original SA reagent, partial damage to biotin-binding sites by fluorophore, and/or inaccurate determination of SA concentration. For routine staining, the mixture of species with maximum tetramer fraction can be used. In cases where the actual

oligomeric form is important, the species of interest can be isolated by gel filtration, although with some loss of material during fractionation (Boniface et al 1998; Cochran & Stern 2000).

III.C.4 *Detection of antigen-specific CD4 T cells in mixed lymphocyte populations*

A major use of MHC oligomers is in identification of antigen-specific T cells in mixed lymphocyte populations. In order to confirm the specificity and sensitivity of oligomer staining, we made a series of dilutions of HA1.7, a DR1-resticted, Ha-peptide specific T cell clone, into a mixture of unstimulated peripheral blood mononuclear cells (PBMCs) from a healthy donor, and stained the mixtures with SA-PE oligomers of DR1-Ha (Fig. III.7). The HA1.7 T cell clone was readily detected at a frequency of 1%. Background staining of the PBMCs was around 0.2%, as detected using DR1 oligomers carrying an endogenous peptide, placing a limit on the sensitivity of this reagent. Similar behavior was observed with other DR1-peptide complexes, and using other PBMCs.

Monocytes exhibited strong non-specific staining by the DR1 oligomers, and can be seen as the large $CD4^{mid}$ population in the center of the profiles shown in Fig. III.7. Staining sensitivity might be improved by exclusion of these cells. This could be accomplished by identifying them by their large FSC/SSC, and gating out cells with these characteristics, but this risks simultaneous exclusion of T cell blasts, which exhibit similar scattering properties. Similarly, gating for low levels of CD4 expression risks losing activated T cells, which might have downregulated their CD4 in response to activation. A better technique is to identify monocytes by surface markers, and we have found CD14-PerCP (Pharmingen) to be ideal for this purpose.

Because of the high background signals observed with class II MHC oligomers, it is important to evaluate the level of non-specific staining carefully in each experiment, particularly if low-frequency populations are under investigation. This can be accomplished best by two different control experiments: staining the experimental T cell population with DR oligomers carrying a control peptide, and staining of a control T cell population with the experimental DR-peptide oligomers. Although the use of control MHC oligomers is not standard practice in class I MHC tetramer staining protocols, we believe that at this stage of technological development, it can provide important information about class II MHC tetramer staining behavior. Endogenous peptides known to be constitutively present, for example TfR for HLA-DR1 (Chicz et al 1992), are particularly suited for use as control peptides, since reactive T cells should have been deleted during negative selection. However, since we have observed heterogeneity among protein batches in their respective background staining levels, it is important to use control T cell populations as well. The identification of an appropriate control T cell population will depend on the details of the experiment and available samples. Using PBMCs from a healthy donor is a reasonable first step, but may not be ideal since their different MHC haplotype and immune state may subtly affect background signals. It is important to note that T cell blasts (recently activated T cells) contribute differently to the background staining than resting T cells, further complicating the identification of an appropriate control T cell population. Since neither control oligomers nor control T cells provide a perfect measure of the non-specific staining, we suggest that both be examined.

Many antigen-specific $CD4^+$ T cell populations of interest are thought to exist in peripheral blood at frequencies lower than 0.2% (Maini et al 1998), and currently such populations represent a challenge for identification using class II MHC oligomers. There has

been a report of direct identification of $CD4^+$ T cells in peripheral lymphocytes using class II MHC oligomers. Meyer and colleagues were able to detect DR4-restricted, OspA-peptide-specific T cells *ex vivo* using DR4 oligomers (Meyer et al 2000). However, these samples were from the inflamed knee of a DR4 homozygous individual with Lyme-disease arthritis, and such a rich source of enriched T cells in such a fortuitous genotype is likely to be the exception, not the rule, for most researchers. We were unable to detect Ha-specific $CD4^+$ T cells in any of multiple PBMC samples tested from healthy, unstimulated donors (data not shown). However, *in vivo* stimulation, either by active infection or vaccination, might be adequate to boost levels above the current detection threshold. Furthermore, antigen-specific T cell frequencies are likely to be significantly different for different pathogens and in various auto-immune states.

III.C.5 *In vitro expansion of antigen-specific $CD4^+$ T cells and detection by MHC oligomers*

Novak and coworkers described a procedure wherein specific PBMCs are expanded *in vitro* in the presence of antigen prior to analysis by MHC oligomers, with proliferation monitored using CFSE (Novak et al 1999). CFSE is a non-specific amine-reactive fluorescein derivative that is stably incorporated into cells and whose signal is diluted two-fold with each cell division. Using this method, we were able to detect the responsive Ha-specific $CD4^+$ T cell populations from two DR1 individuals seven days after initial *in vitro* stimulation (Fig. III.8a,b). Cells in the two left quadrants have divided since the initial stimulation ($CFSE^{low}$). Of these, the oligomer-PE^+ cells (upper left quadrant) are DR1-Ha-specific T cells that have proliferated *in vitro* and are able to bind DR1-Ha oligomer. Although the Ha-specific cells are infrequent, they are above background staining as assessed by oligomers of DR1-TfR. After a single stimulation *in vitro*, the Ha-specific T cells from either donor exhibit 100-fold reduced

CFSE signal, indicating that they underwent 6 or more divisions (the $CFSE^{low}$ signal is close to the auto-fluorescence level and cannot be accurately measured). Very few $CFSE^{low}$ cells were observed in samples stimulated with control peptides (data not shown). An upper-limit for the frequency of the DR1-Ha specific $CD4^+$ T cells in the original samples can be estimated by the CFSE dilution factor and suggests that donor AW22 has fewer than 15 in 100,000, and donor 1H fewer than 3 in 100,000. In our experience, T cell expansions by *in vitro* stimulation varied significantly, even between samples from the same patient, and so these estimates may not be very accurate. However, the identity of these cells was clear, and easily confirmed by secondary stimulation of one of the lines. A second stimulation was performed using irradiated DR1 EBV-transformed B cells pulsed with Ha, and the $CFSE^{low}$, oligomer-PE^+ population was observed to dominate the culture after eleven days (Fig. III.8c).

III.C.6 *Different clones show different temperature dependencies of staining*

We have previously reported that class II MHC oligomer staining of HA1.7 T cells depends on an active cellular response, and that the staining was significantly reduced at low temperatures where membrane rearrangements were blocked (Cameron et al 2001). To investigate the generality of this phenomenon, several different DR1-restricted clones were stained with specific or control SA-PE oligomers for 3 hours at either 4°C, 22°C, or 37°C (Fig. III.9). The clone HA1.7 showed no detectable staining at 4°C (Fig. III.9a), and the clone Cl-1 shows slight staining at 4°C (Fig. III.9b). In contrast, another the DR1-Ha specific clone, HaCOH8 (Fig. III.9c), and a DR1-p24(34) specific clone, AC-25-1 (Fig. III.9d), each showed significant staining at 4°C (>20% of the signal at 37°C). In each of the four clones, staining with DR1 oligomers carrying control peptides resulted in no staining at either temperature (thin

line and dashed line profiles). For a Jurkat T cell lymphoma variant transfected with HA1.7 T cell receptor genes (CH7C17, Fig. III.9e), and for RBL mast cells similarly transfected (Y22.D6, Fig. III.9f, respectively), bright staining by the DR1-Ha oligomers was observed at 37°C but not at all at 4°C, the same behavior as seen in the parental clone HA1.7. Thus, the temperature dependence of class II MHC staining appears to vary significantly clone-to-clone, with some cells only detectable at elevated temperatures, and some cells easily detected at either cold or physiological temperatures.

III.C.7 *Short-term polyclonal T cell lines show heterogeneous staining at 4°C*

The polyclonal lines 1HFB and 1HHA were raised from a DR1 homozygous individual by *in vitro* stimulation with FluB and Ha peptides, respectively, with a first stimulation using autologous APCs, and a second using $DR1^+$ EBV-transformed B cells to restrict the population to those specific (or cross-reactive) with DR1. Each line showed significant staining with appropriate DR1-peptide oligomers when stained at 37°C (Fig. III.9g, 9h, shaded). When stained at 4°C, the cells were less bright, and exhibited a very broad distribution of staining intensities (thick lines). Apparently, these short-term polyclonal lines contain cells with varying abilities to be stained at 4°C. This suggests that $CD4^+$ T cells exhibiting different temperature dependence for class II MHC oligomer staining also exist *in vivo* at significant frequencies.

III.D Discussion

III.D.1 *Summary of various methodologies for production of biotinylated MHC proteins*

The human class II MHC protein DR1 can be produced by expression in *E. coli* of denatured subunits followed by *in vitro* folding in the presence of peptide, and the resultant material has proven useful for a variety of studies including preparation of MHC oligomers and staining of antigen-specific T cells. The protocol is similar to one used to produce the murine class II MHC I-E^k (Altman et al 1993), and has been successfully adapted to the production of DR2a (B5*0101) (Li et al 2000). However, we were unsuccessful in attempts to produce DR3 (B1*0301) or DR4 (B1*0401) by this method (unpublished results). Another researcher has reported single-chain upper-domain-only class II MHC constructs folded from *E. coli* inclusion bodies for other MHC alleles (Burrows et al 1999), but has reported problems with protein aggregation. At present, the *E. coli* expression method can only produce a relatively small set of class II MHC alleles.

For production of a variety of soluble class I MHC alleles, expression in *E. coli* followed by folding *in vitro* (Garboczi et al 1992) is the method of choice, and has been adopted by the NIAID Tetramer Facility (www.niaid.nih.gov/reposit/tetramer/index.html), which currently produces sixteen human, eight murine, six macaque and two chimpanzee class I MHC alleles using this method. There are several major differences between folding class I and class II MHC-peptide complexes. Class I MHC proteins fold in the presence of a mild denaturant (arginine) whereas class II MHC proteins folds only in the presence of a relatively high concentration of a viscous stabilizer (glycerol). Class I MHC alleles fold at relatively high protein concentrations (2 μM), whereas for class II MHC proteins folding proceeds significantly only at low protein concentrations (0.1 μM). Finally, class I MHC subunits can be used "crude," i.e. as solubilized inclusion bodies, while DR1 subunits require purification by

denaturing ion exchange chromatography prior to folding. Whether these differences contribute to the difficulties in generalizing class II MHC folding to other alleles is not clear.

An alternate method of class II MHC production is the use of insect cells to secrete folded MHC protein into the culture medium. This method has successfully produced a number of soluble class II MHC proteins including DR1 (B1*0101) (Stern & Wiley 1992), DR4 (B1*0401) (Kozono et al 1994), DR52a (B3*0101) (J. Gorski, personal communication), I-E^k (Kozono et al 1995), and CD1d (Benlagha et al 2000). Modifications of this approach to enhance MHC subunit assembly, including the use of leucine zippers, chimeric Fc domains, and/or single-chain constructs, have enabled the production of DR2b (Appel et al 2000; Gauthier et al 1998), I-A^d (Rhode et al 1996; Scott et al 1996), I-A^{g7} (Stratmann et al 2000), DQ0601 (Kwok et al 2000), and others. Many researchers also covalently attach their peptides to the N-terminus of the beta chain, but we have found this to be unnecessary for tightly-binding peptides.

Proteins produced from either *E. coli* or insect cells were efficiently loaded with desired peptide. Biotinylation was achieved by either chemical or enzymatic modification, each with high yield. Protein produced from any of these was able to stain antigen-specific $CD4^+$ T cells specifically. In general, for the purposes of large-scale protein production, we find expression in *E. coli* to be easier than insect cells, however this must be weighed against the fact that currently many more class II MHC alleles can be produced in insect cells. In either case, we find the experimental effort to be significantly greater than for the production of class I MHC proteins. For new researchers in the field, the choice of methodologies for production of class II MHC-peptide complexes will depend on the particular alleles of interest and previous experience in protein expression.

III.D.2 *Interpretation of class II MHC oligomer staining results*

We were able to stain a variety of T cell clones and TCR transfectants using oligomers of DR1-peptide complexes. The staining of polyclonal lines further suggests that these reagents are capable of reacting with at least a large fraction of T cells carrying TCRs specific for the DR1-peptide being used. However, it is not clear that all antigen-specific T cells will be detected by this methodology. In Figure III.8, each T cell expansion contains some $CFSE^{low}$ cells which fail to stain with DR1-Ha oligomers. Although this may be due to bystander proliferation, or response to a different MHC allele, it may also include antigen-specific T cells refractory to oligomer staining. Additionally, it has been reported that anergized $CD4^+$ T cells don't stain with DR-peptide oligomers (Cameron et al 2001), possibly due to CD3 downregulation, or to perturbations in activation pathways which might alter TCR clustering and/or internalization. Our results suggest while many $CD4^+$ T cells can be stained by their cognate class II MHC oligomers, looking at the exceptions to this behavior may be especially insightful. Although there is an extensive body of literature suggesting that class I MHC tetramers detect the nearly all $CD8^+$ T cells, there have also been several reports of $CD8^+$ T cells specific for particular peptides which couldn't be stained by cognate MHC oligomers (Bertoletti 2001; de Visser et al 2000; Moser et al 2001; Spencer & Braciale 2000). On the other side of the coin, it is possible that DR-peptide oligomers may prove useful for identifying antigen-specific T cells lacking regular proliferative or effector functions (such as cytokine secretion or cytotoxicity) that are required for other enumeration procedures. Such populations have been identified using class I MHC oligomers on $CD8^+$ T cells (Goulder et al 2000; Welsh 2001). Considering the relative paucity of investigations of $CD4^+$ T cells using class II MHC

oligomers, we cannot yet be certain what types of cells will and won't be detectable by class II MHC oligomers. This should continue to be a focus for researchers in the field.

III.D.3 *Temperature sensitivity of staining; MHC oligomers as probes of T cell Avidity*

We observed substantial differences in the staining behavior of class II MHC oligomers at 4°C and 37°C. Differential staining at cold and warm temperatures has been previously reported for class I MHC oligomers (Whelan et al 1999), although the difference is smaller than that described here for class II MHC oligomers. Moreover, there has been no report of $CD8^+$ T cells incapable of being stained at 4°C but which can be stained at 37°C, behavior which we have observed for several clones and transfectants (Fig. III.9). For both $CD4^+$ and $CD8^+$ T cells, elevated temperatures have been shown to facilitate the internalization of MHC oligomers (Cameron et al 2001; Whelan et al 1999). Thus, staining at 37°C presumably reflects both surface and internalized oligomers, whereas staining at 4°C reflects only surface-bound oligomers.

The bimolecular affinity between an MHC-peptide and TCR is likely to play an important role in determining the ability of a particular T cell to be stained by MHC oligomers (Crawford et al 1998). Temperature effects on MHC-peptide/TCR affinity are not well understood. Crystal structures suggest the docking of two static, relatively flat surfaces (Ding et al 1999; Garcia et al 1996). In this case, enthalpic terms would be expected to dominate the affinity and to favor tighter binding and lower temperatures. However, Willcox and coworkers found evidence for a large entropic factor in the binding of two different class I MHC/TCR pairs (Willcox et al 1999). Although in their system, higher affinities were still favored by lower temperatures, the existence of the large entropic effect suggests the possibility that some

of the difference between clones could be due to differential segmental flexibility in the binding surface of different TCRs. The relationship of such phenomena to the differential temperature sensitivity is currently not clear, and potentially may vary from clone to clone. However, the TCRs from the clones HA1.7 and HaCOH8, which exhibit extremely different abilities to be stained by oligomers of DR1-Ha at 4°C, have been shown to have very similar affinities for DR1-Ha (1.7 μM and 1.8 μM, respectively) (Stone et al 2001). Thus, for at least these two clones, affinity is not the only factor determining their ability to be stained by class II MHC oligomers.

The avidity of MHC-peptide oligomers for cell surface TCR is another parameter worthy of examination. The avidity of a multivalent ligand for a cell surface receptor results from a combination of bimolecular affinity and the accessibility of nearby receptors for crosslinking. We have previously used a model for this reaction that parameterizes avidity as the dissociation constant, K_d, and a crosslinking or oligomerization constant, K_x (Perelson & Delisi 1980). Using this model, we determined that HA1.7 and HaCOH8, although they had similar K_ds, differed in their K_xs by more than 10-fold, with HaCOH8 being the "easier to crosslink" clone (Stone et al 2001). The increased crosslinkability (higher K_x) could be a consequence of static phenomena resulting in shorter distances between receptors, for example as a result of pre-clustered receptors or a local enrichment within lipid microdomains. Alternately, the increased crosslinkability could be the result of a more dynamic phenomenon, including greater receptor freedom of movement, active cytoskeletal involvement, or faster membrane recycling in these cells. Fahmy and coworkers used the K_d and K_x formalism to evaluate naïve and memory T cells from a 2C TCR transgenic mouse reactive for murine class I MHC (Fahmy et al 2001). They observed higher K_x values for the memory cells, suggesting a

crosslinkability difference similar to the one we observe between HA1.7 and HaCOH8. In that report, different K_x values were interpreted to result from changes in the static receptor oligomerization state. We would suggest that T cells could modulate the K_x by either the static or dynamic phenomena discussed above. Several other recent studies have reported alteration of TCR avidity for various $CD8^+$ T cells (de Visser et al 2000; Hesse et al 2001; Margulies 2001; Slifka & Whitton 2001). The short term polyclonal $CD4^+$ T cell lines studied here contain cells of high, low, and intermediate avidity. Avidity modulation may be an important way the immune system regulates T cell reactivity, and we believe that it will be of significant interest to try to understand the physiological causes and consequences of differences in K_x and TCR avidity between between individual clones or their varied activation states.

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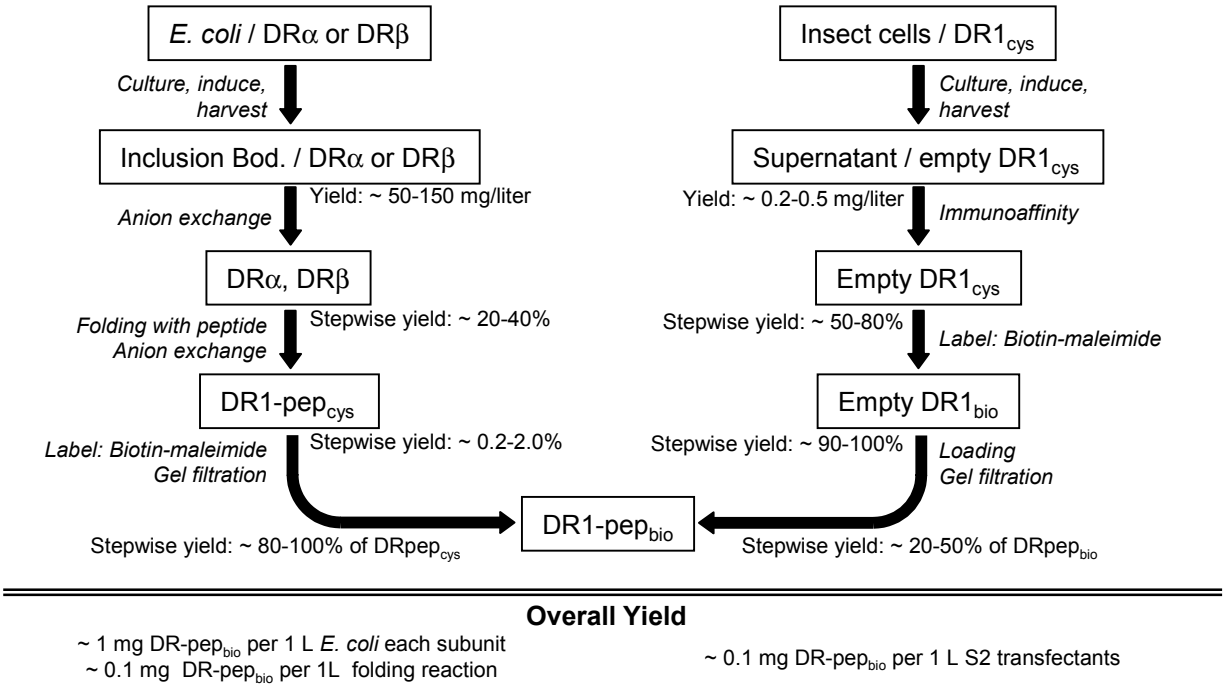


Figure III.1. *Production of biotinylated class II MHC.* DR1 can be produced either from *E. coli* (left panel) or insect cells (Sf9/baculovirus, or S2, right panel). Yield of MHC protein and experimental effort required are similar in each system. Biotinylation can be achieved enzymatically using BSP and biotin-ligase with similar ease and efficiency to the chemical modification shown here. So far, only DR1 (B1*0101), DR2a (B5*0101), and I-E^k have been produced from *E. coli* by folding *in vitro*. Several protein modifications to facilitate chain pairing or problematic alleles have been applied in the insect cell system, including introduction leucine zippers, single chain constructs, and chimeric Fc domains. These methods have not been thoroughly investigated in the *E. coli* system. Covalent peptide attachment to the β chain would eliminate the peptide loading step in either system, but is not necessary for tightly binding peptides.

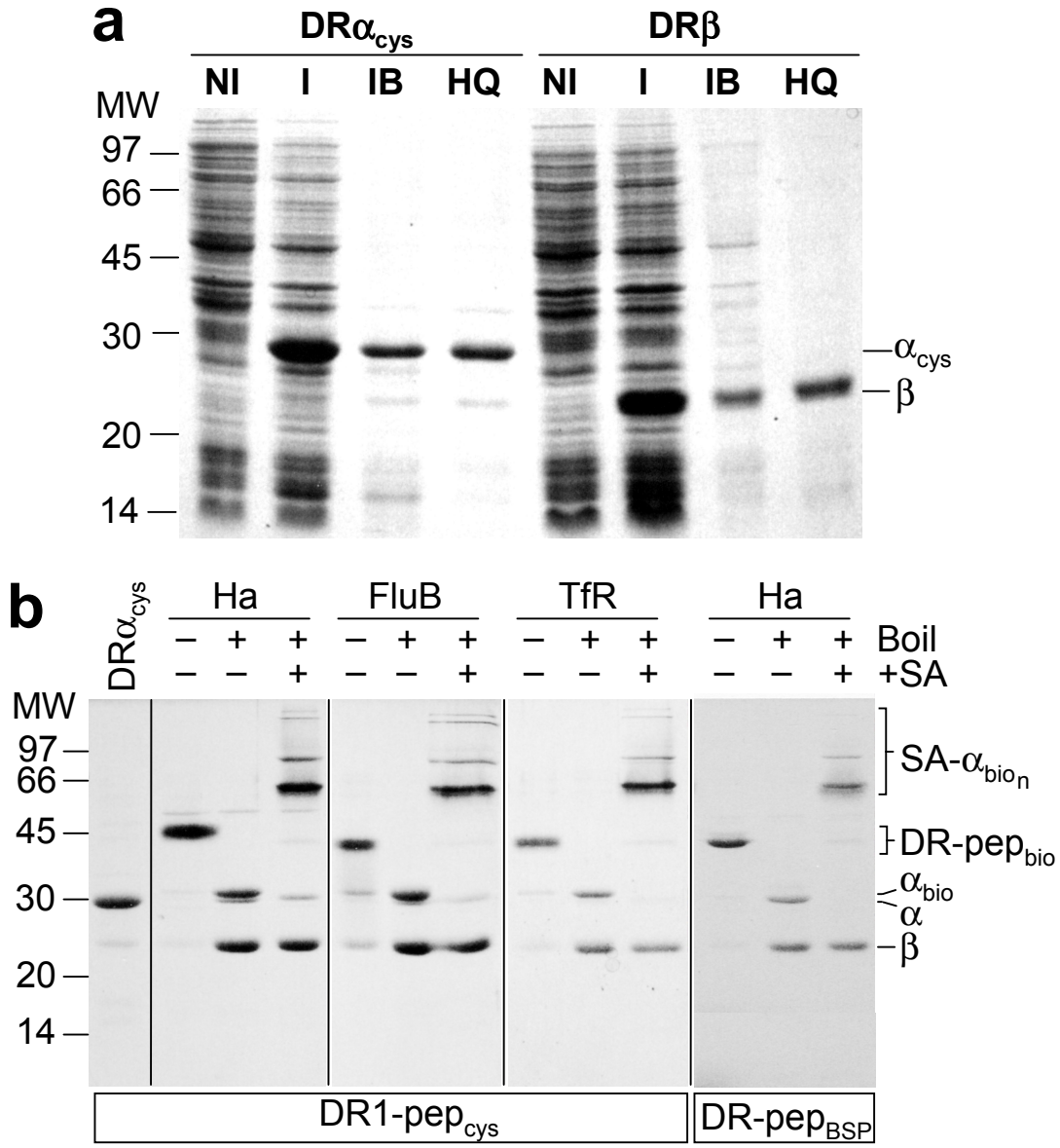


Figure III.2. *SDS-PAGE analysis of class II MHC expression, purification, folding, and biotinylation.* (a) SDS-PAGE of *E. coli* lysates before (NI) and after (I) induction, isolated inclusion bodies (IB), and purified subunits (HQ). BL21 DE3 *E. coli* were previously transformed with either pLMI DRα_{cys} (lanes 1-4) or pLMI DRβ (lanes 5-8). (b) SDS-PAGE of DR1-peptide-biotin complexes. Each section consist of equivalents amounts of DR1-peptide$_{cys\text{-}biotin}$ that had not been boiled, boiled for 2 minutes, or boiled, chilled, and supplemented with excess SA. Efficient peptide loading is indicated by the migration of $\alpha\beta$ heterodimer (DR-pep$_{bio}$) complexes in the not-boiled lanes. Efficient biotinylation is demonstrated by the disappearance of α_{bio} after the addition of SA and the formation of SA-α_{bio^n}. Biotinylation can also be observed for the αcys constructs by the small gel shift between α_{cys} and α_{bio} bands. The first lane contains DRα_{cys} inclusion bodies. Positions of molecular weight markers (kDa) are indicated on each gel. Gels contained 12.5% acrylamide and were stained with Coomassie brilliant blue.

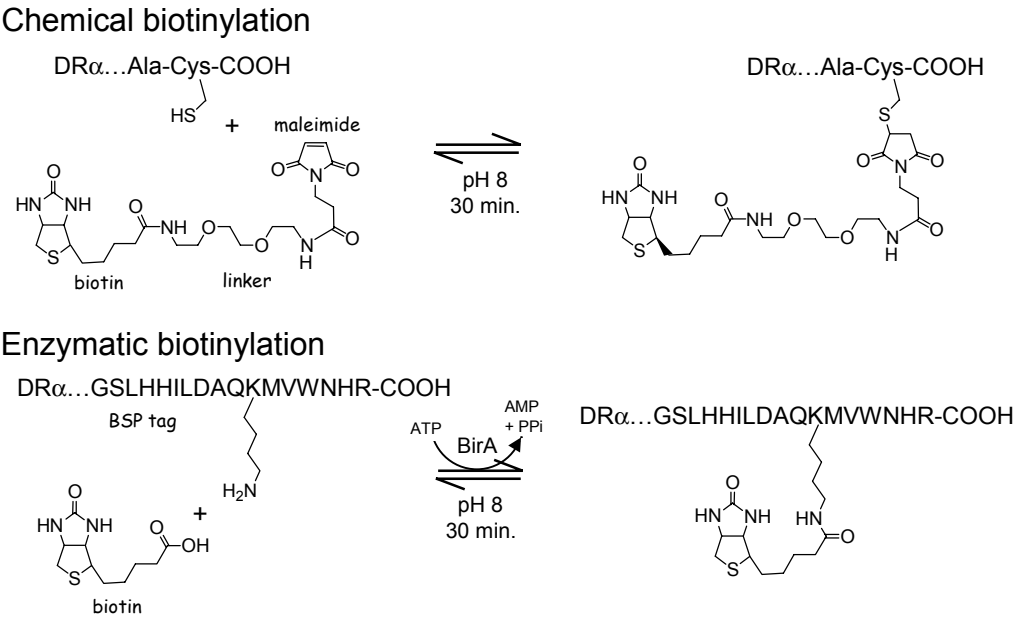


Figure III.3. *Biotinylation strategies for class II MHC. Both enzymatic and chemical biotinylation can be achieved with high efficiency and high yield.* (a) The sulfhydryl of a cysteine side-chain can be specifically modified by maleimide reagents, such as the water soluble biotin-maleimide reagent with a 29 Å linker as shown. (b) Proteins carrying a suitable biotin-ligase substrate peptide (BSP) can be enzymatically biotinylated by biotin ligase (BirA) in the presence of ATP.

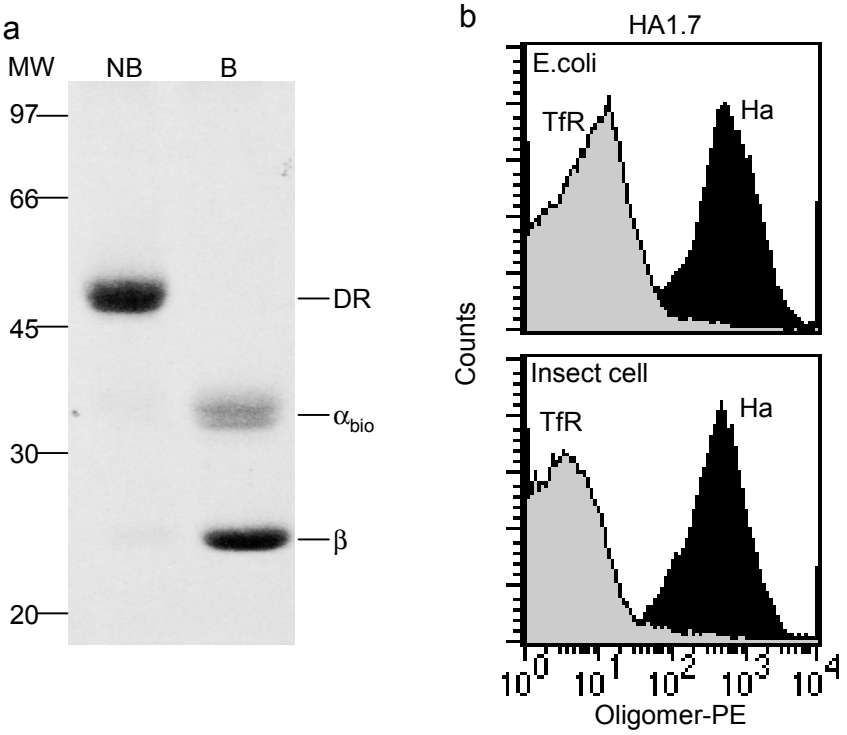


Figure III.4. *DR1-peptide complexes produced in insect cells.* (a) As an alternative to expression in *E. coli,* $DR1_{cys}$ was expressed in S2 insect cells, purified, biotinylated, loaded with Ha peptide and analyzed by SDS-PAGE as not boiled (NB) or boiled (B). Quantitative peptide loading is indicated by the absence of any free α or β subunits in the NB sample. (b) Staining efficiencies of DR1 produced from *E. coli* and from S2 cells are comparable. HA1.7 T cells were stained at 37ºC with SA-PE oligomers of DR1-Ha (black) or DR1-TfR (gray) from *E. coli* (upper panel), or from S2 cells (lower panel).

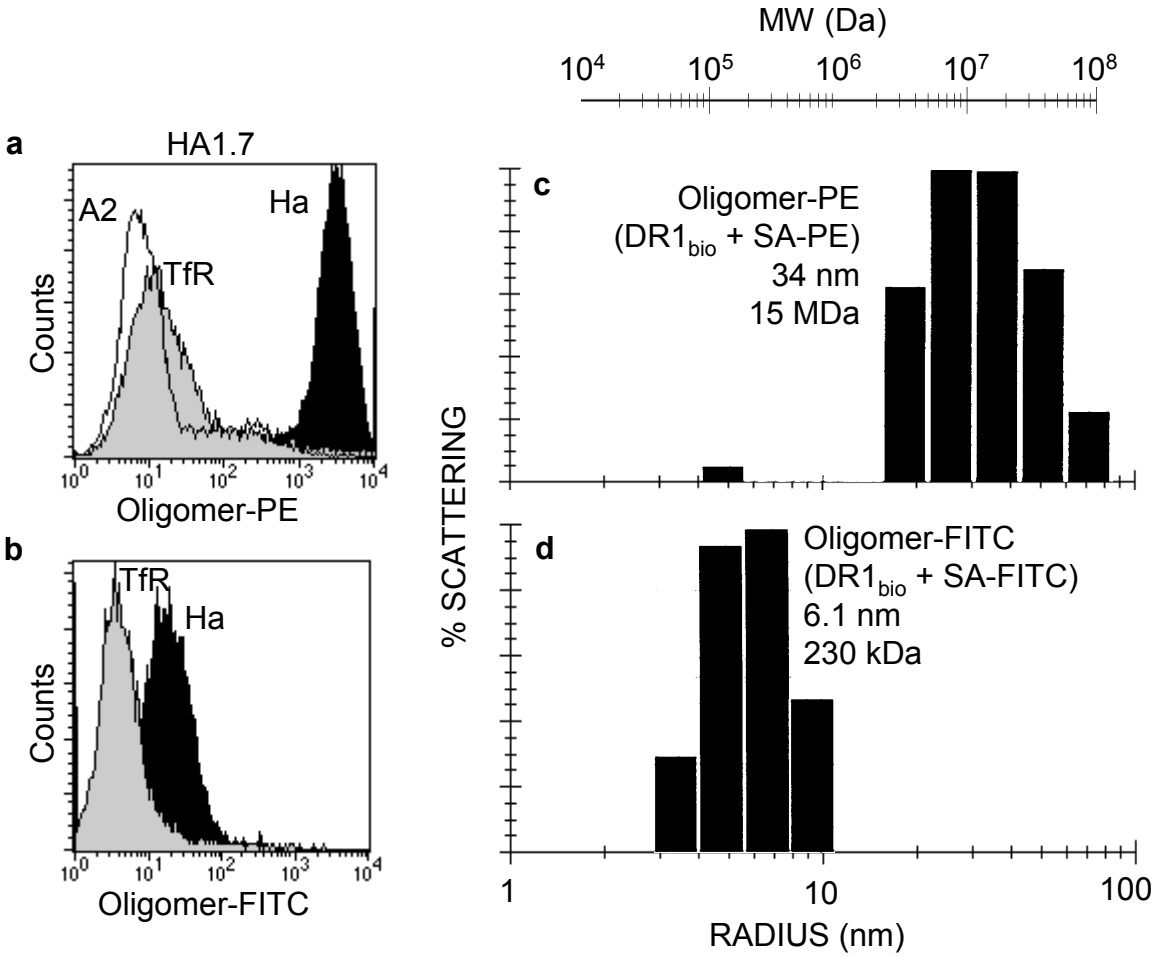


Figure III.5. *Comparison of DR oligomers formed with SA-PE and SA-FITC.* (a)(b) Comparison of the staining of the different oligomers. HA1.7 T cells were stained at 37ºC with SA-PE (a) or SA-FITC (b) oligomers of DR1-Ha (black), DR1-A2 (unfilled), or DR1-TfR (gray). DR1-Ha oligomers formed with SA-PE (a) were found to be much brighter than tetramers formed with SA-FITC (b). (c)(d) Hydrodynamic radii of different oligomers were estimated by dynamic light scattering. (c) DR1-SA-PE oligomers exhibited mean radii of 34 nm, corresponding to a molecular weight of 15 MDa, consistent with large oligomeric forms. (d) DR1-SA-FITC oligomers exhibited mean radii of 6.1 nm, corresponding to a molecular weight of 230 kDa, consistent with a ratio of 3 or 4 DR1 bound to one SA. Mean radii were converted to approximate molecular weight values using a standard curve model for globular proteins. Superior brightness of SA-PE reagents is probably due to both the greater sensitivity of PE vs FITC in flow cytometry, and to the higher valency of SA-PE oligomers.

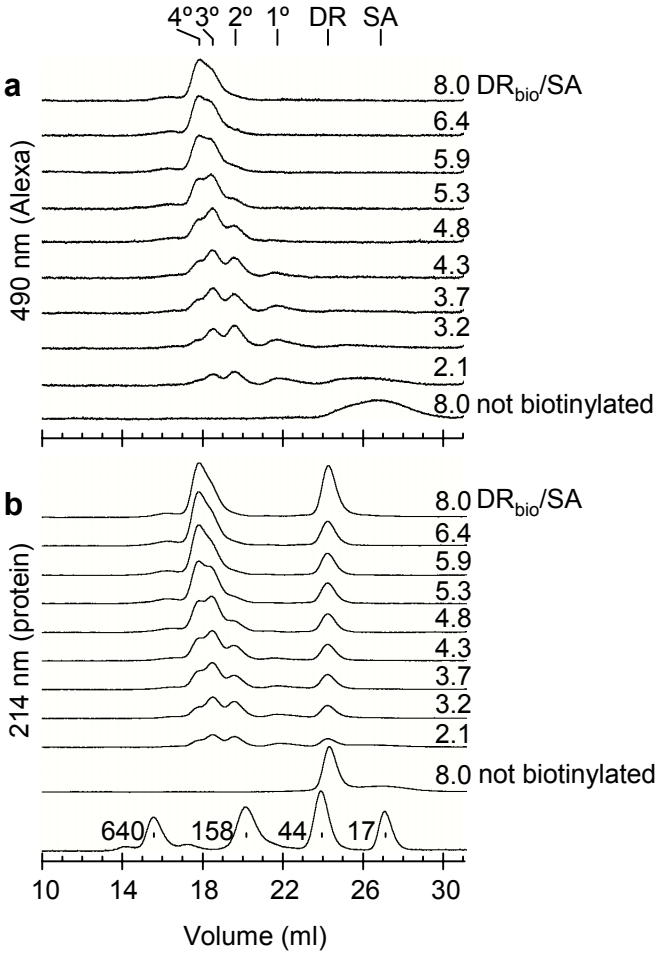


Figure III.6. *Oligomerization of DR1-pep$_{bio}$ by SA-Alexa.* Gel filtration chromatography was performed on mixtures of DR1-TfR-biotin and SA-Alexa, and monitored at (a) 490 nm and (b) 214 nm, using two columns in series. Molar ratios of DR to SA are indicated at the right of each trace. The bottom trace in each panel is SA-Alexa mixed with non-biotinylated DR1-Ha. Elution positions for oligomers and monomers are marked at the top of (a). A trace of molecular weight standards is shown as the last trace of (b). Uncomplexed SA-Alexa absorbs to the gel filtration matrix and thus elutes at a smaller apparent molecular weight than expected.

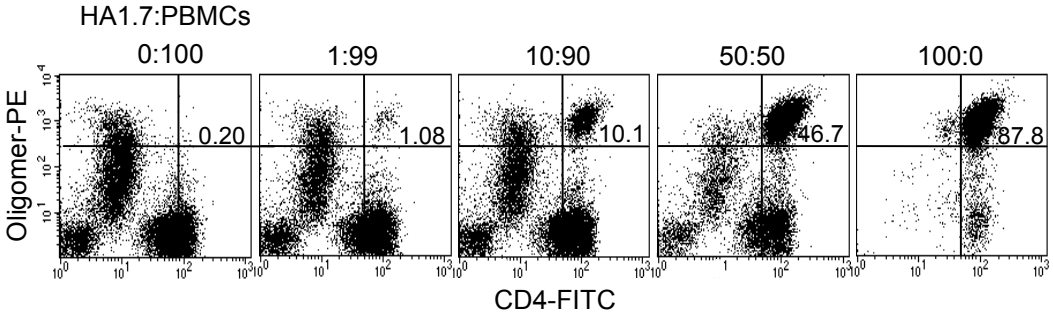


Figure III.7. *Determination of detection limit in mixed lymphocyte populations.* In order to assess the potential for detection of antigen-specific $CD4^+$ T cells in fresh PBMC samples, a clone of known specificity (HA1.7) was diluted into non-specific PBMCs at the ratios shown above each plot. Cells were stained with SA-PE oligomers of DR1-Ha at 37°C, co-stained with α-CD4-FITC at 4°C, and analyzed by flow cytometry. Percentage of cells $CD4^+$ PE^+ is indicated in the upper-right quadrant of each plot. Specific $CD4^+$ T cells were readily detected at a 1:99 frequency, but detection below this limit may be difficult. Monocytes, identified by $CD4^{low}$ and FSC^{large} phenotype and observed in the center of the plots, bound non-specifically to the DR1 oligomers, regardless of peptide used, suggesting that sensitivity might be improved by removal of this population using differential adhesion, or exclusion by co-staining a monocyte-specific marker such as CD14.

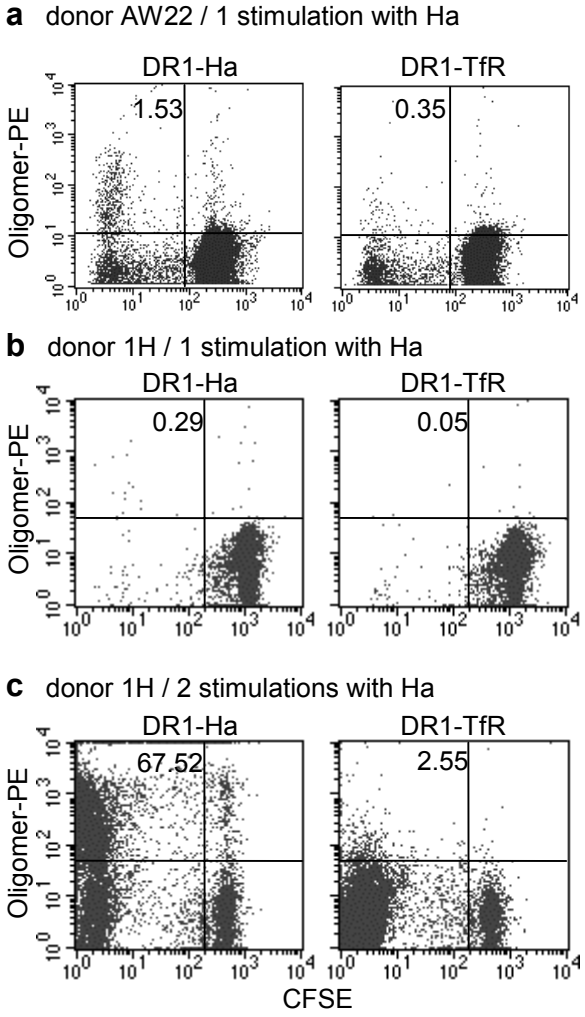


Figure III.8. *Short-term in vitro expansion of PBMCs to detect antigen-specific CD4$^+$ T cells*. Since Ha-specific CD4$^+$ T cells were undetectable in the fresh PBMCs of multiple donors (data not shown), cells were expanded by *in vitro* stimulation with Ha peptide. (a,b) After one stimulation, Ha-specific T cells could be detected. (c) After a second stimulation, the culture was dominated by Ha-specific CD4$^+$ T cells in the CFSE^{low}, Oligomer-PE$^+$ quadrant (upper left) of the DR1-Ha stained samples. PBMCs from two DR1 donors, (a) AW22, and (b,c) 1H, were stained with CFSE, stimulated with 5 μM Ha, and stained seven days later with DR1-Ha or DR1-TfR at 37°C. The 1H culture was stimulated a second time by 5 μM Ha presented by DR1 EBV B cells, and stained eleven days later with oligomers of DR1-Ha or DR1-TfR at 37°C. Each plot was gated for live, CD4$^+$ cells. (Exclusion of the oligomer-binding monocyte population observed in Fig. 7 was unnecessary as very few survived the culture conditions). Cell proliferation is shown by low CFSE staining. The percent of CFSE^{low} (proliferating), PE$^+$ (antigen-specific) cells is indicated in the upper left quadrant of each plot.

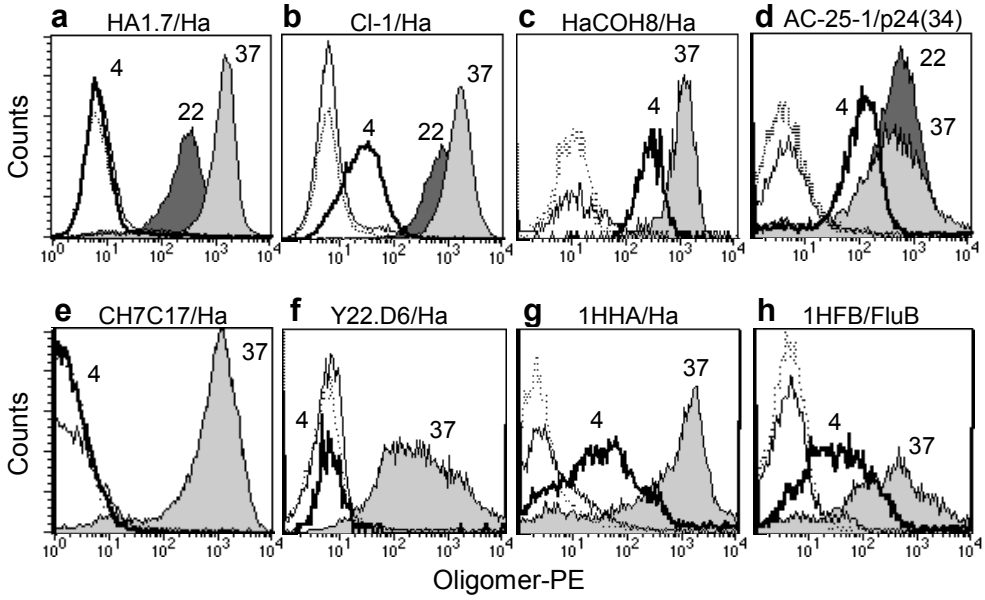


Figure III.9. *Oligomer staining of T cell clones, transfectants, and short-term polyclonal lines, at different temperatures.* Some $CD4^+$ T cells can be detected only when stained at 37ºC, while others can be readily identified at either 4ºC or 37ºC. (a-h) Cells were stained at 37ºC with SA-PE oligomers of DR1-Ha (shaded trace) or DR1-TfR (thin trace) or at 4ºC with SA-PE oligomers of DR1-Ha (thick trace) or DR1-TfR (dashed trace). Selected clones were also stained with DR1-Ha oligomers at 22ºC (dark shaded trace). Each panel is labeled with the name of T cell line or clone / antigenic peptide. (a-c) CD4 T cell clones specific for DR1-Ha. (d) CD4 T cell clone specific for DR1-p24(34). (e) Jurkat T cell derivative transfected with HA1.7 TCR. (f) RBL cells transfected with HA1.7 TCR. (g)(h) Polyclonal T cell lines raised by two *in vitro* stimulations of $DR1^+$ PMBCs against either FluB (g) or Ha (h) peptide.

IV. Towards TCR Proteomics: Examination of a highly diverse repertoire of $CD4^+$ T cells specific for an influenza peptide bound to HLA-DR1

We have combined several recent technological advances in immunology and molecular biology to allow us to identify and sequence of a large number of TCRβ genes specific for a particular peptide. We utilized MHC tetramers and IFN-γ surface capture to identify and isolate $CD4^+$ T cells specific for the Ha peptide derived from influenza hemagglutinin and restricted by HLA-DR1. After total RNA was extracted from these pools, we made a "tagged" first-strand cDNA pool using Smart cDNA^{TM} technology and amplified all TCRβ genes simultaneously regardless of Vβ usage. Hundreds of clones from this amplified pool were isolated and sequenced. Detailed analysis of these sequences, derived from three different patients, reveals an extremely diverse repertoire, with little overlap between patients. Although there is no clear motif in the CDR3 regions of these sequences, most of the clones appear to utilize acidic residues in CDR1 and CDR3, presumably to interact with the basic residues in the Ha peptide. We argue that these results are one of several recent examples of a burgeoning field of large-scale TCR repertoire analysis.

IV.A Introduction

At the center of adaptive immunity is the vast repertoire of ligand specificities contained within the antigen-binding sites of immunoglobulins and T cell receptors. In the last two decades immunologists have learned an enormous amount about how antigens are processed presented to T lymphocytes, how the T cells are triggered, and the consequences of their activation, but relatively little is known about the rules governing which T cells will respond to

which epitopes, how HLA haplotype affects the T cell repertoire, why some epitopes are more immunodominant than others, why disease pathology can differ from person to person, and, ultimately, how we can manipulate these processes to build immunity or suppress autoimmunity.

T cell receptor diversity results from complex genetic rearrangements during development. There are two sources of the receptor diversity: permutational diversity from the pairing of individual gene segments from a pool of germline candidates, and additional N-region diversity at the junction of these gene segments by the random addition and deletion of nucleotides. The TCRα chain is composed of one variable (V) segment and one joining (J) segment. The TCRβ chain is composed of one V segment, one D segment (occasionally 2 or 3), and one J segment. At the TCRα locus there are 42 V and 61 J candidate genes. At the TCRβ locus there are 48 V, 2 D, and 13 J candidate genes. N-region diversity occurs at the V(D)J junction region called the complementarity-determining region 3 (CDR3). CDR1 (contained within the V segment) and CDR3 are known to directly contact the antigenic peptide bound to MHC molecule (Hennecke & Wiley 2001).

Several methodologies have been developed to analyze the repertoire of T cells responding to particular pathogens, proteins, or epitopes. The majority of these studies examine the extent of skewing of either Vβ usage or CDR3 lengths compared to normal distributions caused by monoclonal or oligoclonal T cell expansions (Vβ mAb staining, spectratyping, CDR3 length polymorphism analysis). However, it is difficult for these methods to quantitate T cell diversity on a clonal level when the responding population is polyclonal. The most immunodominant and successfully-attacked epitopes may well be the ones with the broadest response, exhibiting the least skewing. One such epitope is Ha, a highly conserved

epitope from the hemagglutinin protein of A-strain influenza viruses, for which more than thirty reactive TCRβ sequences have been published and which appear to be relatively unrelated to each other (Hennecke et al 2000).

Clonal analysis of antigen-specific T cells typically involves the *in vitro* isolation and maintenance of individual T cell clones. This process is labor intensive and technically difficult, and may not accurately reflect the *in vivo* responding repertoire (Annels et al 2000; Prevost-Blondel et al 1995). Recent advances in the identification of antigen-specific T cells, MHC tetramers (Altman et al 1996), and cytokine surface-capture (Brosterhus et al 1999), have enabled immunologists to isolate clones after minimal manipulation outside of the patient. We have utilized these technologies to sort out populations of $CD4^+$ T cells reactive for the Ha epitope derived from the hemagglutinin protein of A-strain influenza viruses. From these sorted pools, we isolated a TCRβ cDNA library using cDNA technology that enabled the simultaneous amplification of all TCRβ genes with minimal sequence-dependent bias. Direct sequencing yielded an extremely large and diverse set of TCRβ sequences, all specific for a single peptide, Ha, and restricted by a single MHC, HLA-DR1.

Here we present the Ha-reactive, DR1-restricted TCRβ sequence data that we have generated and examine it in order to elucidate the structural factors involved in recognition of the Ha epitope. We argue that this and other recent work (Bourcier et al 2001; Cohen 2001; Hennecke & Wiley 2001; Naumov et al 1998) form the basis of an emerging field of TCR proteomics.

IV.B Materials and Methods

IV.B.1 Peptides

Ha [306-318] (PKYVKQNTLKLAT), FluB [308-320] (PYYTGEHAKAIGN), p24(34) [34-46] (PEVIPMFSALSEG), and TfR [680-696] (RVEYHFLSPYVSPKESP) were synthesized using solid-phase Fmoc chemistry, and purified by C18 reverse-phase HPLC. Both peptides bind tightly to DR1 with dissociation constants below 100 nM (Zarutskie et al 1999).

IV.B.2 *Preparation of fluorescent class II MHC oligomers*

Our methodology for the production of fluorescent oligomeric DR1-peptide reagents has been described in detail previously (Cameron et al 2001). Briefly, truncated versions of the HLA-DR1 α and β subunits were expressed in *E. coli*, purified from inclusion bodies, and folded *in vitro* in the presence of peptide as previously described (Frayser et al 1999). Soluble DR1 peptide complexes were chemically biotinylated on a free cysteine at the c-terminus of the α subunit using maleimide-oxyethylene-biotin (Pierce Chemical Co.). Excess biotin reagent was removed by gel filtration chromatography or extensive dialysis, and DR1-peptide oligomers were formed by stepwise addition of streptavidin-R-phycoerythrin conjugate (SA-PE, Biosource, Inc.).

IV.B.3 T cell culture

Short-term polyclonal T cell lines were raised by *in vitro* stimulation of lymphocytes isolated from $DR1^+$ volunteers. Freshly isolated PBMCs were aliquoted into 24-well plates at 5 million lymphocytes per well in RPMI with 10% human serum, 50 units/ml penicillin G (Gibco), and 50 μg/ml streptomycin sulfate (Gibco), and stimulated by the addition of 5-20 μM

peptide. Starting on day 7, IL-2 (40 units/ml, Aldesleukin, Chiron Corp., Emeryville, CA) was added to the media every 3-4 days. Further *in vitro* stimulations of short-term T cell clones were performed every 2-3 weeks using an irradiated mixture of non-autologous peripheral blood lymphocytes and a DRB1*0101 EBV-transformed B cell line (EBV1.24) that had been pulsed with 1 μM Ha peptide. Every 3-4 days, 40 units/ml interleukin-2 was added to the cultures. The polyclonal lines 1HHA and HA03 were derived from the same volunteer with Ha peptide. The 1HFB line was derived from the same individual but with the FluB peptide. Unlike the other T cell lines, HA03 was raised from CD8-depleted PBMCs and was stimulated with autologous mitomycin-C-treated lymphocytes every week for the first four weeks. After this point, it was stimulated as described above.

A Jurkat T cell mutant selected for lack of T cell receptor (TCR) expression and subsequently transfected with genes coding for the HA1.7 TCR, called CH7C17 (Hewitt et al 1992; Wedderburn et al 1995), was passaged in RPMI supplemented with 10% fetal bovine serum, penicillin G, streptomycin, L-glutamine, 400 μg/ml hygromycin, 4 μg/ml puromycin.

IV.B.4 DR1 oligomer staining

T cells (~10^7/ml) were mixed with DR1-peptide oligomers for 3-5 hrs. at 37°C, chilled for 5 minutes, supplemented with appropriate secondary antibodies for 30 minutes, and washed twice with cold wash buffer (PBS, 1% fetal bovine serum, 15 mM sodium azide). Secondary antibodies used were anti-CD4-APC (Diatec, Norway), and various anti-TCRVβ-FITC (Beckman-Coulter or Pierce Chemical Co.). In some analytical experiments, samples were fixed with cold 1% paraformaldehyde in PBS and stored overnight for analysis the next

morning. The oligomer-stained T cells were analyzed on a FACSCalibur™ flow cytometer or isolated on a FACSVantage SE™ cell sorter (Becton Dickinson).

IV.B.5 Activation and INF-γ surface-capture assay

EBV-transformed DR1 homozygous B cells (EBV1.24) pulsed with 1 μM Ha peptide and mixed with the expanded WL1, WL2, or SL populations at a 4 to 1 ratio and a final concentration of 10^7 cells/ml. Antibodies against CD28 and CD49d (Becton-Dickinson) were added to 1 μg/ml to augment the stimulation. After 6-9 hours at 37˚C in 5% CO_2 the INF-γ capture assay was performed following the manufacturer's protocol (Miltenyi Biotec). Briefly, the cells were washed with cold PBS/1% FCS and resuspended at 10^8 cells/ml in cold media, and the INF-γ surface-capture antibody was added. The capture reagent is a dual-specificity polyvalent antibody complex made by chemically cross-linking anti-CD45 mAb to anti-IFN-γ mAb. This reagent will bind to all lymphocytes through its anti-CD45 arm regardless of activation. After five minutes on ice the cells were diluted with warm media to 10^6 cells/ml, and placed in a 37˚C incubator for 45 minutes with gentle mixing every 5 minutes. Only T cells that are actively secreting IFN-γ will capture significant amounts of IFN-γ due to its relatively high local concentration before the cytokine diffuses into the media. The cells were then centrifuged, washed, resuspended in PBS/1% FCS and incubated with anti-CD3-Fluorescein (FITC), CD4-Allophycocyanin (APC) (Becton Dickinson), and 7-aminoactinomycin (7AAD, Molecular Probes) for ten minutes on ice. An anti-IFN-γ-Phycoerythrin (PE) antibody (Miltenyi Biotec) was added and the cells were incubated an additional ten minutes on ice. The cells were then washed and labeled with magnetic beads coated with an anti-PE antibody at 15˚C for ten minutes and magnetically sorted (Miltenyi

Biotec). The bead sorted T cells were analyzed on a FACSCalibur™ flow cytometer or further purified on a FACSVantage SE™ cell sorter (Becton Dickinson).

IV.B.6 PCR Amplification, Cloning and Sequencing

Total RNA was extracted from the ag-specific T cell pools using Trizol reagent. First strand DNA synthesis was accomplished using a downstream primer derived from near the end of the TCR Cβ gene (AATCCTTTCTCTTGACCATG) and Superscript II reverse transcription (RT) (Gibco). A hybrid RNA/DNA primer from SMART™ cDNA kit (Clontech) was used to attach a common upstream anchor peptide to all first strand DNA molecules in order to enable simultaneous and unbiased amplification of all TCRβ cDNA regardless of their Vβ sequence. PCR amplification was done using the Advantage cDNA PCR Kit (Clontech) following the manufacturer's SMART PCR protocol using a Clontech primer for the upstream primer, and a downstream primer derived from near the beginning of the TCR Cβ gene (TTGGGTGTGGGAGATCTCTGCTTCTGATGGC). PCR products were purified from agarose gels and cloned using the TOPO™ TA cloning kit (Invitrogen) into TOP10 *E. coli* and hundreds of colonies were isolated. Plasmids were generated from individual colonies as needed and DNA sequencing was done at the Massachusetts General Hospital DNA sequencing core facility. Some of the returned sequences were ignored due to containing uninterpretable data.

IV.B.7 Statistical Analysis

The statistical comparison of two or three pools of TCRβ sequences or Vβ usage distribution was accomplished by two methods: determination of the correlation coefficient, r

(done pairwise for three-way comparisons), and chi-squared analysis (Bevington & Robinson 1992; Rosenthal & Rosnow 1991). Results from chi-squared analysis of the data sets were provided as several parameters: χ^2, the value of a chi-squared function performed on the data sets; DF, degrees of freedom, (# of rows -1) * (# of columns -1); p, the one-tailed probability of the chi-squared distribution at the appropriate degrees of freedom; N, total number of observations. For analysis of Vβ usage, unrepresented TCR Vβ genes were included in the comparison up to 30 total genes (29 or 58 degrees of freedom for a comparison of two or three data sets, respectively).

IV.C Results

IV.C.1 Polyclonal T cell lines can be stained with DR1 oligomers

The polyclonality of Ha-specific DR1-restricted T cells was assessed by staining with DR1-Ha "tetramers" and mAbs against TCR Vβ families. The use of soluble fluorescent oligomers of DR1-peptide complexes to stain antigen-specific $CD4^+$ T cells has been described in detail previously (Cameron et al 2001). As a positive control sample we used a modified Jurkat T cell line stably transfected with the HA1.7 TCR which stained brightly with DR1-Ha oligomers (Fig. IV.1a). HA03 and 1HHA are two polyclonal T cell lines from the same individual but each specific for the Ha peptide and restricted by HLA-DR1. Most of the cells in each line (87% and 80%, respectively) were Ha-specific and DR1-restricted, as evidence by staining with DR1-Ha oligomers (Fig. IV.1b,c). Similarly, most of the cells (80%) of a line derived from the same individual against the FluB peptide stained with DR1-FluB oligomers (Fig. IV.1d). None of these lines showed appreciable staining when reacted with oligomers of DR1 carrying non-specific peptides (data not shown). The non-staining cells (Fig. IV.1b,c,d,

lower two quadrants) are most likely non-specific cells which have survived in culture through bystander stimulation, alloresponse versus the non-autologous PBMCs used to stimulate, or activation mediated by peptides in the non-autologous serum or transformed B cell line.

The use of monoclonal antibodies (mAbs) with differing specificities for TCR Vβ families in flow cytometry experiments are a standard technique for the analysis of the repertoire of T cell receptors in particular samples (Diu et al 1993). We combined this methodology with MHC-oligomer staining in order to estimate the Vβ distribution of several short-term T cell lines. Analysis of CH7C17 by this method correctly identifies it as a monoclonal population carrying Vβ3 TCR (Fig. IV.1e). The polyclonal lines HA03 and 1HHA are Ha-specific, DR1-restricted lines derived from the same individual but at different times and initially by different stimulation schemes (see Methods). The oligomer-staining populations of these lines exhibit significantly different Vβ usage patterns as assessed by Vβ-specific mAb binding. HA03 appears to be comprised largely of T cells bearing Vβ13.1/.3 TCRs (78%, Fig. IV.1f). 1HHA appears to contain T cells carrying several different families of Vβs: Vβ1, Vβ3, Vβ5.2/.3, Vβ11, Vβ13.1/.3 (Fig. IV.1g). The FluB-specific, DR1-restricted line 1HFB appears to be largely Vβ13.1/.3 (38%, Fig. IV.1h), although many cells are unaccounted for in the analysis. This indicates that polyclonal populations of cells can be detected by oligomer staining, and that the Ha peptide bound to DR1 can be recognized by TCRs from multiple Vβ families.

IV.C.2 TCRβs can be analyzed in detail by sorting, cloning and sequencing

The Vβ-specific mAb methodology has severe limitations. It provides no information about the degree of polyclonality within a Vβ family, and specific mAbs do not exist for some TCR Vβs. In order to overcome these problems, we combined several recent technological advances in immunology and molecular biology. We use MHC oligomers or cytokine surface-capture assay to identify and isolate antigen-specific T cells, employ cutting-edge cDNA technology to make an unbiased TCRβ cDNA library, and sequence large numbers of clones using inexpensive large-scale DNA sequencing facilities. This methodology is summarized in Figure IV.2, and has been used previously to analyze $CD8^+$ T cells with success (Cohen 2001). Because we were unable to detect Ha-specific T cells using either IFN-γ surface-capture or DR1 oligomer staining in fresh PBMC samples (data not shown), we stimulated PBMC samples *in vitro* with the Ha peptide in order to amplify the frequency of Ha-reactive T cells. In order to maximally preserve the T cell diversity to the Ha antigen, lines were only been expanded *in vitro* once.

The most error-prone step in our methodology is the PCR amplification, which may skew the populations towards certain easy-to-amplify sequences. In our approach, first strand DNA synthesis was accomplished with a downstream TCR Cβ-specific consensus primer (Fig. IV.2b). Because TCRVβ sequences preclude the use of an upstream consensus primer, we utilized a hybrid DNA/RNA primer technology commercially available from Clontech (SMART™ cDNA technology) that adds an "anchor" sequence to the upstream end of the first strand DNA. Thus, using this technology we were able to amplify all TCRβ mRNAs simultaneously and with identical primers. Using this methodology, we previously analyzed a bulk $CD8^+$ T cell population (antigen non-specific) and observed extremely high diversity, exemplified by broad distribution of TCR Vβ usage, and extremely low frequency of any single

TCRβ sequences (Cohen 2001). This increases our confidence that our methodology probes the TCRβ repertoire with minimal bias, although we cannot exclude the possibility of differential skewing between samples.

IV.C.3 Ha-specific T cells can be identified in once-stimulated PBMCs by DR1-Ha oligomer staining

PBMCs from three $DR1^+$ individuals were stimulated *in vitro* with Ha peptide and expanded for three weeks with the addition of IL-2 during the second and third weeks. Figure IV.3 shows that DR oligomer staining determined that a significant portion of the expanded lines were Ha-specific. Expanded populations were analyzed using oligomers of DR1 carrying control peptide (TfR, top row) or specific peptide (Ha, bottom row). Oligomer-staining cells were detected only in the DR1-Ha oligomer samples. The expanded populations of WL1, WL2 and SL were observed to contain 7%, 5%, and 8% Ha-specific DR1-restricted T cells, respectively (data not shown for WL2 because cells were exclusively used for sorting and not analyzed in analytical experiments due to a relatively low total number of cells). $Oligomer^+$ $CD4^+$ live T cells from the WL1, WL2 and SL Ha-expanded samples were sorted by flow cytometry and used to make total mRNA for TCRβ amplification, cloning and sequencing.

IV.C.4 Ha-specific T cells can be identified by IFN-γ secretion

Antigen-specific T cells within the expanded population of individuals WL1 and SL were identified using a commercially available IFN-γ surface-capture kit. This analysis was not performed on WL2 due to the small number of cells in this sample. This surface-capture methodology was chosen instead of the more common technique of intracellular IFN-γ staining

because it enabled identification of the cells without chemical fixation that will damage the TCRβ mRNA. Figure IV.4 shows that this method was able to sensitively detect Ha-specific DR1-restricted $CD4^+$ T cells within the expanded populations of individuals WL1 and SL. Cells were stimulated with either control peptide (TfR, top row) or specific peptide (Ha, bottom row). CD3-low $CD4^+$ T cells were only detectable in the samples treated with Ha peptide (Fig. IV.4b,d, lower right quadrants). IFN-γ secreting cells were detected only in Ha-treated samples (Fig. IV.4f,g, upper right quadrants). IFN-γ-secreting $CD4^+$ live T cells were sorted by flow cytometry and used to make total mRNA for TCRβ amplification, cloning and sequencing. Dot plots of CD3 vs. IFN-γ show that the CD3-low population is identical to the IFN-γ-secreting population (data not shown), suggesting that the identification of antigen-specific cells might be similarly accomplished by analyzing the $CD3^{low}$ population.

IV.C.5 A large number of different TCRβ sequences found in DR1-Ha specific $CD4^+$ T cells

$CD4^+$ T cells specific for Ha and restricted for DR1 were detected and isolated by either DR1 oligomer staining (Tet pool) or IFN-γ surface-capture staining (IFN-γ pool). From these cells, total mRNA was isolated, TCRβ cDNA were amplified and cloned into *E. coli*, from which hundreds of colonies were isolated and individual plasmids were prepared and DNA sequenced. The resultant sequencing data was compiled and is summarized in Table IV.1. Some samples were ignored due to containing uninterpretable data. Vβ gene usage was assigned according to the nomenclature of Arden, et al., 1995. For each sequence the Vβ, CDR3 and Jβ used are indicated. The CDR3 region is at the site where Vβ, Dβ and Jβ gene segments were joined during T cell maturation, and is therefore the most diverse of the three

CDR regions, having resulted from the permutational diversity of joining different V, D and J segments augmented by random nucleotide deletion and addition.

Out of 270 TCRβ sequences collected, 95 unique TCRβ sequences were observed. Fifty of them were observed only once, but 45 of them occurred more than once, either within one of the pools (44 sequences) or in both of the pools (12 sequences). The highest frequency sequence observed was SL-G-A40, which was observed 16 times in the DR1-oligomer pool (Tet) and 5 times in the IFN-γ pool, for a total of 21 observations out of 111 total sequences (19%) from volunteer SL.

Notably, there is only one TCRβ sequence that appeared in more than one individual; WL2-T-E52 occurred twice in the DR1-oligomer sorted pool (Tet) of patient WL2 and has the same protein translation as SL-G-A54, which occurred twice in the IFN-γ sorted pool (IFN-γ) of patient SL. Every other TCRβ sequence observed was unique to that individual. In contrast, many of the sequences appeared in both the Tet and IFN-γ pools of a single individual (61 of 120 observations in WL1, 57 of 111 observations in SL). The most notable exception to this rule is WL1-T-D1 which appeared 10 times in the Tet pool of WL1, but not even once in the IFN-γ pool. Because all ten of the observations of WL1-T-D1 occurred from the same PCR amplification experiment, and no WL1-T-D1 sequences were observed in another similar PCR amplification reaction, this suggests some degree of skewing in the repertoires during amplification. Therefore, in most of our analyses, sequences are analyzed without weighting by their occurrence frequency (unweighted). In some analyses we analyzed the sequences both with and without weighting.

IV.C.6 None of these TCRβ sequences match previously published data

Table IV.2 shows a list of TCRβ sequences previously published from Ha-specific DR-restricted T cell clones. None of these sequences are identical to ones we have newly identified here. However, a cursory glance at the Vβs used suggests some similarity between the previously published sequences (Vβ3.1, Vβ11.1, Vβ13.1) and our new sequences from WL1, WL2 and SL.

IV.C.7 Comparison of Vβ mAb analysis with Sequences isolated directly

In order to confirm our results from the direct sequencing approach shown in Figure IV.2 we compared the Vβ usage of our sequences with Vβ usage as determined by anti-TCR Vβ mAb analysis. Figure IV.5 shows a comparison of Vβ usage in patients WL1 (a) and SL (b). Direct sequencing data was analyzed without weighting by the number of occurrences of particular sequences. A moderate correlation is observed between the Vβ mAb analysis and the direct sequencing data for WL1 (r=0.58). A strong correlation is observed between the Vβ mAb analysis and the direct sequencing data for SL (r=0.94). Sources of error in the Vβ mAb analysis include clonal distribution shifts during the intervening time between direct sequencing analysis and Vβ mAb analysis, difficulty staining T cells with Vβ mAbs after DR1-oligomer staining due to CD3 downregulation, variable brightness of different Vβ mAbs, and poorly defined or imperfect Vβ specificities of the mAbs. Our direct sequencing approach is subject to skewing during PCR amplification. For these reasons, we suggest that the correlations observed are within experimental error and argue that most of the TCRβ sequences we have identified by direct sequencing are valid with no gross bias introduced by our methodology.

IV.C.8 Some TCRβ sequences contain similar CDR3 regions

A ClustalW algorithm (Thompson et al 1994) was used to compare the CDR3 regions of our newly identified TCRβ sequences (Table VI.1) and the previously published ones (Table IV.2). Results were plotted as phylogenetic trees in order to assess the similarities between patients (Fig. IV.6). When color-coded by patient, relatively little patchiness is observed on the tree (Fig. IV.6a), indicating no gross differences between individuals. However, small clusters of sequences can be observed within individuals and for WL1 in particular (see the clusters of blue dots marked by asterisks). This may be caused by differential deletion of T cell clones during development caused by the individuals' other MHC alleles, or by different infection histories.

Table IV.3 shows the top 0.7% of the pairwise matches from the ClustalW analysis (scores > 70) organized by families of related sequences. From our newly identified TCRβ sequences, nearly half of them have a close relative within this data set (42 of 95 unique sequences, 127 of 270 observations). A similar fraction of the previously published sequences appear to have close relatives within the data set (14 of 32). Differences between adjacent sequences are highlighted in bold. Many sequences differ from another by as little as a few homologous residues (e.g., WL1-G-D41 and WL1-T-B18 differ only by S-to-A and E-to-D mutations, and B103 and WL2-T-B68 differ only by G-to-D and D-to-E mutations). Others differ quite significantly and might not have been identified as related without the use of the algorithm.

IV.C.9 Statistical analysis suggests that most of the TCRβ sequences are reliable

In order to further analyze the reliability of the new TCRβ sequences as being genuinely derived from Ha-specific, DR1-restricted T cells, we divided the sequences into approximately two equal sized groups, one set of "validated" sequences considered to be more reliable, and the remaining non-validated sequences. We assigned a sequence to the validated group according to two criteria: if the sequence appeared in both the Tet and the IFN-γ pools of the individual, or if the sequences exhibited significant homology to another sequence within the data set or previously published sequences (Table IV.3). Any sequence not validated was assigned to the non-validated group. Using this logic, 45 of 95 unique sequences and 178 of 270 observations were "validated." These results are summarized in Table IV.4. After making the assumption that the validated sequences were genuine Ha-specific DR1-restricted TCRβ sequences, and because antigens typically stimulate a skewed pattern Vβ usage, we figured that if the non-validated sequences exhibited similar Vβ usage when compared to the validated sequences then they were likely to be mostly comprised of genuine sequences. In Table IV.4 the Vβ usage distribution of the two groups were compiled and statistical parameters were derived from their comparisons. Moderate to strong correlations were observed between the validated and non-validated groups within the Tet pool, IFN-γ pool, or both pools together. Two points can be drawn from this analysis: moderate overall correlations suggest that most of the non-validated sequences are genuine, however the Tet pool appears to be less reliable than the IFN-γ pool.

IV.C.10 Particular Vβ genes are highly utilized by the DR1-Ha-binding repertoire

Now that we have validated the majority of the TCRβ sequences listed in Table I, we can analyze the data set in order to understand which characteristics of these sequences appear

to be critical for interacting with DR1-Ha. In Table IV.5 we show the distribution of Vβ usage within each individual, both with and without weighting. We performed chi-squared analysis on individual Vβ frequencies and compared them to the expected frequency of Vβ genes if Vβ selection were completely random (1 in 48) and have indicated any significant deviations ($p<0.05$) in bold. Vβ usage is graphically analyzed in Figure IV.7. No single Vβ gene appears to be used significantly by all three individuals. Vβ3.1 is highly utilized by both WL1 and WL2. Vβ1.1 is highly utilized by WL1 and SL. Vβ11.1 is highly utilized by WL2 and SL. Overall, the Vβ genes 1.1, 3.1, 11.1 and 13.1 appear to be the most commonly utilized genes for binding DR1-Ha. Many functional Vβ genes are entirely absent from both the new and previously published sequences (listed at the bottom of Table IV.5).

Table IV.6 shows the occurrences of different Vβ genes within each individual and the results of a statistical analysis comparing all three individuals to each other. The correlation coefficient between WL1 and WL2 Vβ usage indicated moderate-to-strong correlation ($r=0.68$, 0.69 in weighted and unweighted data sets). However, both WL1 and WL2 correlate moderate-to-weak with SL ($0.34\leq r\leq 0.45$ for each comparison). Chi-squared analysis indicate that the differences in the unweighted set are not statistically significant ($p=0.40$), but are significant in the weighted set ($p<0.01$). This may be a consequence of inaccurate weighting caused by skewing during the PCR amplification step. Thus, we can only conclude that there are similarities in the Vβ usage between the patients, and that there may be significant differences as well. This agrees with the slight patchiness observed in the phylogenetic tree analysis (Fig. IV.6). The differences in Vβ usage between individuals may be an imprint from lymphocyte

development caused by negative selection against the other class II MHC alleles of the individuals' haplotype.

We also compared the Vβ distribution of our data set with the previously published Ha-reactive TCRβ sequences (Table IV.7). Because there are so few DR1-restricted Ha-specific TCRβ sequences in the literature, we compared only to the whole set of Ha-specific DR-restricted sequences. There is a moderate correlation between the two sets (r=0.61, 0.72 for the weighted and unweighted sets). The differences, however, appear to be significant (p=0.01 in the weighted set, p=0.09 in the unweighted set). The most obvious culprit of this difference is Vβ 3.1 which comprises only 18% of the DR1-restricted set, but is 58% of the overall set in the literature. This suggests that our new TCRβ sequences do not differ significantly from the previously published Ha-specific TCRβs with the exception of the extremely strong preference of other DR alleles for Vβ3.1.

IV.C.11 DR1-Ha binding TCRβ sequences use acidic residues in CDR1 and CDR3

Because the Ha peptide contains three lysines, all of which appear to be accessible for TCR contact (Stern et al 1994), and because the structure of the HA1.7 TCR binding to DR1-Ha shows contacts between two of the lysines and the TCRβ chain, we hypothesized that Ha-specific TCRβ sequences might be enriched for negatively charged residues in their CDR regions. Figure IV.8 shows a detailed analysis of the charge distributions in all three CDRs of the sequences in Tables IV.1 and IV.2. Indeed, in both the IFN-γ and Tet pools, all three CDRs have negative average net charges (Fig. IV.8a). This data, however, should be compared to the average charges expected from random sampling of all 48 functional Vβs. This analysis shows

that in fact, within CDR2 the newly identified TCRβ sequences appear to be less negatively charged than the set of all 48 Vβs. This is not unexpected, since in all known TCR-MHC crystal structures, only the CDR1 and CDR3 regions contact the antigenic peptide, while the CDR2 region contacts only the MHC (Hennecke & Wiley 2001). Examination of CDR1 reveals that the average charge of the 48 Vβ genes in this region is only –0.1, while that of our data set is –0.8. The average charge of previously published Ha-specific DR1-restricted sequences is similar (-1.3). The average charge of all previously published Ha-specific sequences is significantly higher (-2.6) reflecting a heavy reliance on Vβ3.1 (-3 charge in CDR1) by non-DR1 restricted Ha-specific TCRβs sequences.

Similarly, our Ha-specific DR1-restricted TCRβ data set exhibits a negative average charge in the CDR3 region (-1.1 in either the weighted or unweighted sets). However, since this region is formed by V-D-J recombination and is highly diverse, it is difficult to know what the average charge in this region of an unbiased (not Ha-specific) data set would be. Nonetheless, it is remarkable that 90% of the unique sequences have at least one negative charge, that 73% have a net negative charge, only 31% of them contain any positive charges, and only 6% have no charges. Notably, HA1.7, for which there is a crystal structure in complex with DR1-Ha, has no charges in its CDR3 region but still manages to contact the two lysine residues through serine and threonine residues.

To examine the charge characteristics of the CDR3 region of our TCRβ sequences more carefully, we calculated the percent of negative and positive charges at twelve positions starting at either end of the region. Results are shown in Figure IV.8b,c. Because the first few residues of the CDR3 region are often contributed by the C-terminus of the Vβ gene, and the last few residues of the CDR3 can be contributed by the N-terminus of the Jβ gene, the frequency of

charged residues in these genes is plotted at either end of the alignment (open circles). Alignment at the beginning of the region shows no significant bias towards Vβ genes with negative charges to contribute to the CDR3. However, alignment at the end of the CDR3 regions reveals that 50% of the CDR3 sequences have a negative charge at position N-3 caused by a bias towards Jβ segments 1.1, 2.1, 2.2 and 2.7.

In addition, there appears to be a preference for negative charges in the intervening region between the Vβ and Jβ segments (positions 8-12, N-5, N-6). This is not due to biased D gene usage since none of the functional D genes in either direct or inverted orientation have a net negative charge. Thus, Ha-specific DR1-restricted TCRβ genes appear to be biased for negative charges introduced by N-region diversity during TCRβ recombination. HA1.7 TCR doesn't contain any charged residues in its CDR3 region, although only 96% of sequences in Table IV.1 contain at least one charged residue. This suggests a potentially different binding mode for most of the TCRs identified here than in the HA1.7/DR1-Ha crystal structure (Hennecke et al 2000).

IV.C.12 TCRβ sequences detected by DR-oligomer staining and IFN-γ secretion are similar.

We compared the TCRβ sequences detected by DR1-oligomer staining with those detected by IFN-γ secretion within individuals WL1 and SL in order to see if the two methods preferentially detected certain T cells more than others (Table IV.8). Vβ usage was compared and in both volunteers the two pools correlated with each other ($r>0.6$) and differences did not appear to be significant ($p>0.3$). We further compared the pools with each other on a clone-by-clone basis. In this analysis, omission of the weighting factors led to negative correlation

coefficients. This is because the sequences which appear in both the Tet and IFN-γ pools also tend to be high frequency sequences, suggesting that at least some portion of the weighting reflects frequency in the original samples. When weighting is included, correlations are moderate-to-weak ($r=0.4$ for WL1, $r=0.36$ for SL) and differences appear to be significant ($p<0.01$ for both). One of the sequences contributing to this difference is WL1-T-D1 which appears 10 times in the Tet pool of WL1, and zero times in the IFN-γ pool. However, the WL1 Tet pool sequences were derived from cDNA made in two different PCR reactions, and all ten of the WL1-T-D1 occurrences arise from one of these two reactions, strongly suggesting that they were the result of biased amplification. We are thus unable to conclude that the differences between the Tet and IFN-γ pools are significant as they may be the result of sample-to-sample PCR amplification differences. We can at least conclude that there is some similarity between the two pools. Further experimentation will be required to investigate the potential differences between these detection methodologies.

IV.D Discussion

The methodology utilized here and in another recent paper (Cohen 2001) overcomes some of the previous challenges in the field of TCR repertoire analysis: it analyzes the TCR repertoire at the clonal level through direct sequence of the TCR chains (for TCRα analysis see (Cohen 2001)), and it can be used successfully on mixed T cell populations directly from the patient (Cohen 2001) or after minimal *in vitro* expansion. The methodology can be utilized for populations of varying degrees of oligo- or polyclonality simply by scaling the number of

clones isolated, made feasible for large data sets by the continuing deflation of automated DNA sequencing prices.

We have here presented an extremely large set of TCRβ sequences specific for a single antigen, Ha, and restricted by a single MHC allele, HLA-DR1. It is possible, even likely, that some of these sequences may be spurious, not Ha-specific. This might have arisen from poor T cell sorting, or cross-contamination during PCR. However, several arguments suggest to us that the vast majority of these sequences are genuinely Ha-specific: the Vβ usage of WL1 and SL is similar to the Vβ usage determined by mAb Vβ analysis; the overall Vβ usage of our data set is similar to the Vβ usage previously observed in the 1HHA line, and in previously published Ha-specific T cell clones; some sequences appeared in both Tet and IFN-γ pools; some sequences appear to be related to other sequences within the data set or among the previously published sequences; these validated sequences have a similar Vβ usage to all of the remaining non-validated sequences in our data set. Nonetheless, we would advise that the most highly reliable Ha-specific DR1-restricted TCRβ sequences are the ones that we deem validated, those which either appeared in both the Tet and IFN-γ pools (see Table IV.1), or appear to be related to other sequences of the set (see Table IV.3).

The weighting of clone frequency according to the number of occurrences within our data set may be more problematic. It is clear that we have no gross error with weighting as evidenced by no single clones having entirely dominated our data. Furthermore, the Tet/IFN-γ overlapping sequences are most likely to have been high frequency clones in the original samples, and tend as well to have higher weighting factors in our analysis, suggesting that our weighting does indeed have some significance. However, one clone, WL1-T-D1, appeared ten times in one single round of amplification, and never appeared in any other analysis from that

same patient, strongly indicative of either cross-contamination of that sample or significant skewing of the repertoire during the PCR amplification. As well, correlation coefficients from the comparison of various data sets within this paper consistently exhibit slightly lower correlations for the weighted data sets than for the unweighted ones, further suggesting experimental error in the weighting. In summary, we neither conclude that our weighting is useless, nor is it accurate. We would advise that the best judge of a high frequency clone is that it appears in both the Tet and IFN-γ pools. Future work utilizing this methodology should analyze sequences from as many independent amplification reactions as possible in order to minimize this skewing problem.

Detailed examination of the Ha-specific TCRβ sequences suggest several insights into the recognition mechanism utilized by the immune system for this particular antigen, and possibly for others as well. One clear conclusion is the absence of a "public TCR" for DR1-Ha; many different TCRβ sequences, derived from many different Vβ and Jβ genes and with widely divergent CDR3 regions, are capable of interacting with DR1-Ha. Previously examined antigen-responding T cell populations have exhibited either heavy usage of only one or a few Vβ families, and/or contained strong CDR3 motifs (Acha-Orbea et al 1988; Bourcier et al 2001; Moss et al 1991; Naumov et al 1998; Utz et al 1996).

One common mechanism of binding can be gleaned from the data -- the use of acidic residues in the CDR1 and CDR3 regions of the TCRβ chain. This is almost certainly in order to interact with the three solvent-exposed basic residues of the Ha epitope. T cell activation does not require especially high bimolecular affinity, but depends instead on extremely high ligand selectivity, and the charge-pair contacts may be able to be understood in this way, leading us to an intriguing hypothesis; that because it is easier to achieve highly specific

binding of a charged surface than a hydrophobic one, the exposed hydrophilicity and/or charge of an epitope might correlate with the breadth of the responding TCR repertoire and possibly with immunodominance. Since the above idea about specific binding and electrostatic interactions is a relatively speculative and controversial one, we direct the reader to (Noskov & Lim 2001; O'Shea et al 1992; Sheinerman et al 2000).

Although the above hypothesis is highly speculative, it begins to suggest the potential of a thorough and detailed TCR proteomics research program. As much as we understand of antigen presentation and T cell activation, we still lack the ability to explain why some peptides stimulate effective immune responses and others do not, or in some individuals but not in others. Large amounts of research is performed on a small handful of T cell clones in order to understand the mechanism of peptide antagonists, or to develop peptido-mimetics, but with very limited understanding of the TCR repertoire that will eventually encounter such antigens when they are eventually used clinically. A detailed structural understanding of the TCR repertoire and the selective forces under which it develops would invaluably aid our understanding of disease pathogenesis and the development of new vaccines. But because the repertoire is so vast, and antigens so diverse, such knowledge is likely to be gained only from approaches similar to the ones being developed in the fields of genomics, proteomics, and bioinformatics.

We believe that the work presented here in conjunction with other recent studies (Bourcier et al 2001; Cohen 2001; Hennecke & Wiley 2001; Naumov et al 1998) represent a step towards this goal of detailed TCR repertoire analysis through large-scale proteomic analysis. The Ha antigen is of particular interest because it represents a reasonable ideal; a promiscuous DR-binding epitope (Brawley & Concannon 1996; O'Sullivan et al 1991;

Zeliszewski et al 1996) with broad recognition by patients ((Gelder et al 1995), and unpublished observations) stimulating a large repertoire of T cells. Further studies of the Ha-responding TCR repertoire, including analysis of the TCRα chain, larger TCRβ data sets, studies on a variety of MHC haplotypes, and structural determination of several more Ha-binding TCRs, will open the door to a new understanding of T cell immunology.

IV.E Acknowledgments

I would like to thank Rebecca Wolfe for help with statistical analysis, Dikran Aivazian and Bader Yassine-Diab for assistance obtaining samples, Glenn Paradis for assistance with flow cytometry, and Lucy Wedderburn for the CH7C17 line and for advice growing T cell lines.

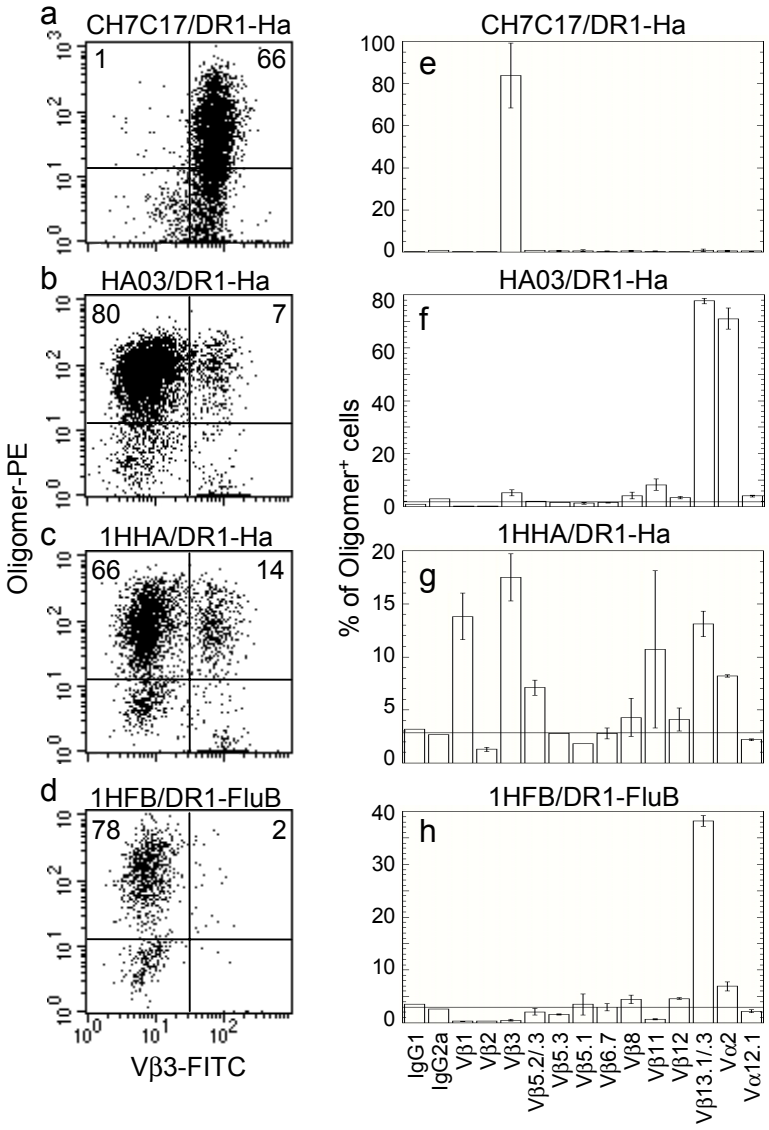


Figure IV.1. *Short-term T cell lines are polyclonal.* T cell lines were stained with oligomers of DR1-Ha and counterstained with antibodies against TCR Vβ or TCR Vα families. Representative dot plots are shown (a-d) and degree of polyclonality in the lines is demonstrated with bar graphs (e-h). (a,e) CH7C17 is a mutant TCRβ-deficient Jurkat T cell line transfected with Ha-specific, DR1-restricted HA1.7 TCR. (b,f) HA03 is a polyclonal line derived from a single individual by repetitive stimulation with DR1-homozygous B cells and Ha peptide. (c,d,g,h) 1HHA and 1HFB are short-term polyclonal lines from the same individual as HA03 raised against Ha and FluB peptides, respectively.

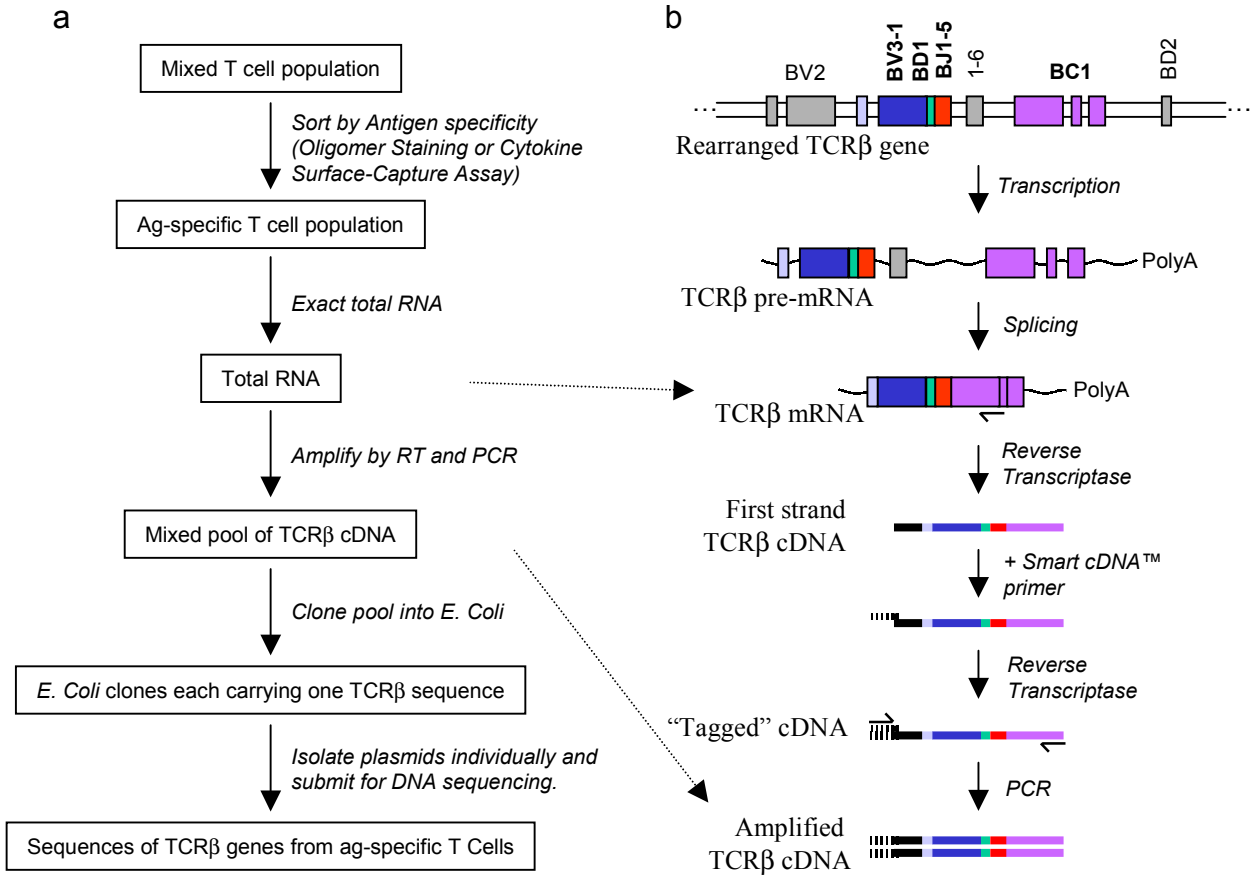


Fig IV.2. *Identification of TCRβ sequences specific for particular MHC-peptide.* Flow charts summarize the experimental procedure for the identification, isolation, amplification and cloning of TCRβ sequences specific for particular peptide antigens. Procedure can be easily adapted for analysis of $CD8^+$ T cells or TCRα. This methodology has been made possible several key technological advances in the last few years: MHC tetramer staining and cytokine surface-capture assay to identify and sort antigen-specific T cells, Smart cDNA™ technology to amplify sequences from few cells (10^4) regardless of TCR Vβ family, and recent drastic reduction in the cost of large-scale DNA sequencing projects caused by the genomics boom.

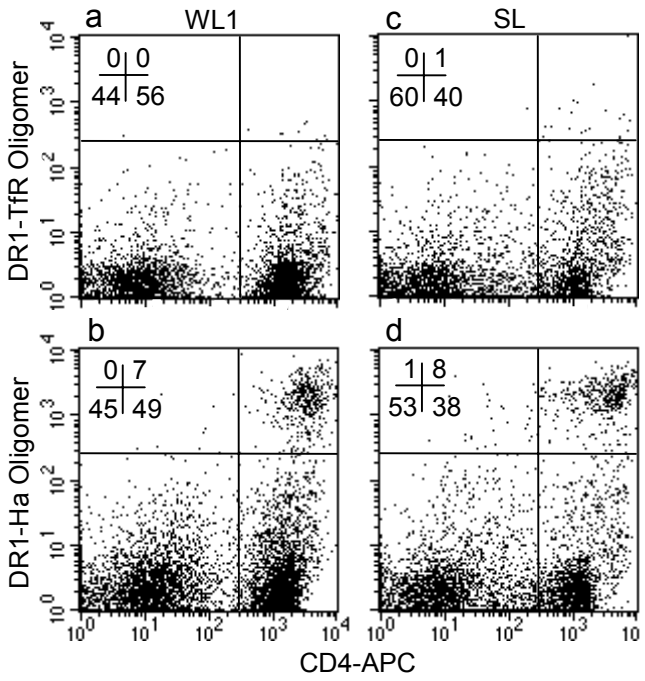


Figure IV.3. *Detection and sorting of Ha-specific DR1-restricted CD4$^+$ T cells.* PBMCs from volunteers WL1 (a,b), WL2 (not shown), and SL (c,d) were stimulated once *in vitro* with Ha peptide, cultured for three weeks and subsequently stained with DR1-TfR oligomers (a,c) or DR1-Ha oligomers (b,d). Percent of cells in each quadrant is indicated in the upper left quadrant of each dot plot. CD4$^+$ T cells specific for Ha peptide are detected in the upper right hand quadrant of panels (b,d). Cells of this quadrant were isolated by FACS sorting and used make total mRNA.

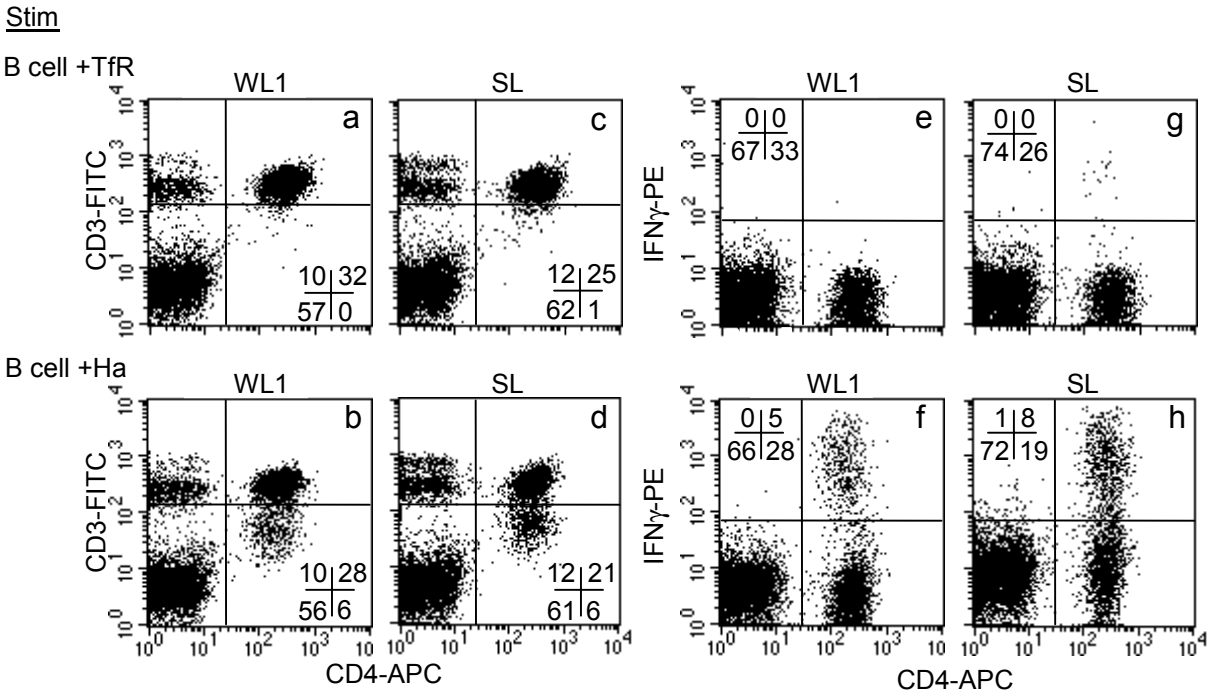


Figure IV.4. *Detection and sorting of Ha-specific DR1-restricted CD4$^+$ T cells.* PBMCs from volunteers WL1, WL2 (not shown), and SL were stimulated once *in vitro* with Ha peptide, cultured for three weeks and subsequently stimulated with DR1-homozygous B cells pulsed with either TfR peptide or Ha peptide. Shortly after the second stimulation, cells were stained for IFN-γ secretion using a surface-capture methodology. Dot plots of CD4 vs. CD3 (a-d) and CD4 vs. IFN-γ (e-h) are shown. Percent of cells in each quadrant is indicated in the lower right quadrant of each plot. CD4$^+$ T cells specific for Ha peptide are detected in the upper right hand quadrant of panels (f,h). Cells of this quadrant were isolated by FACS sorting and used make total mRNA. These cells appeared to be identical to the CD3^{low} cells in the lower right quadrant of panels (b,d), suggesting an alternative isolation strategy.

Table IV.1. *Vβ TCRs specific for DR1-Ha identified by* direct sequencing

Subject	ID	Occurances Tet	IFN-γ	Vβ[a]	CDR3[b]	Jβ
WL1	WL1-G-A35		1	1.1	CASSVGPGPGDTEAFF	1.1
	WL1-G-A14		2	3.1	CASSLISEPNTEAFF	1.1
	WL1-G-A10		2	3.1	CASTQGVLTYEQYF	2.7
	WL1-G-A29		2	3.1	CASSGVPEQTPSYEQYF	2.7
	WL1-G-A37		1	8.1	CASTPSWETEAFF	1.1
	WL1-G-A5		1	8.1	CASSPYRAQNTEAFF	1.1
	WL1-G-A8	2	1	1.1	CASSVVPEWEGELFF	2.2
	WL1-G-A34	3	1	1.1	CASSPLTGGQTDTQYF	2.3
	WL1-G-A7	1	1	3.1	CASSSPSLADNNEQFF	2.1
	WL1-G-A11	3	5	3.1	CASRTGTHTDTQYF	2.3
	WL1-G-D41	1	2	3.1	CASSLSPELETQYF	2.5
	WL1-G-A13	7	11	6.5	CASSVGQGKAFF	1.1
	WL1-G-A18	1	2	6.5	CASSLGQGEQFF	2.1
	WL1-G-A1	7	13	8.1	CASSLSQGDQPQHF	1.5
	WL1-T-D39	1		1.1	CASSVAPSPSETEAFF	1.1
	WL1-T-B20	1		1.1	CASSVAPSGSESPLHF	1.6
	WL1-T-B22	1		1.1	CASSTGFSEQFF	2.1
	WL1-T-D22	4		1.1	CASSAGFSEQFF	2.1
	WL1-T-D1	10		2.1	CSARGQAIYGYTF	1.2
	WL1-T-B10	2		3.1	CASSPGQIYGYTF	1.2
	WL1-T-D12	1		3.1	CASIKGGQDYSPLHF	1.6
	WL1-T-B2	1		3.1	CASSPGGGDTYNEQFF	2.1
	WL1-T-B16	2		3.1	CASSDSGTYNEQFF	2.1
	WL1-T-B13	3		3.1	CASSTIGLDTGELFF	2.2
	WL1-T-B18	4		3.1	CASSLAPELDTQYF	2.3
	WL1-T-D3	1		3.1	CASSLVPELGEQYF	2.7
	WL1-T-D10	1		6.6	CASSEAWTSGKNEQFF	2.1
	WL1-T-E10	1		8.1	CASSSDGTGEKLFF	1.4
	WL1-T-B21	7		11.1	CASSVLTDNGYTF	1.2
	WL1-T-D17	2		13.1	CASSREGTVNHGGYTF	1.2
	WL1-T-D2	5		13.1	CASTGSSYEQYF	2.7
	WL1-T-B11	1		13.1	CASSSPWETQYF	2.7
	WL1-T-D8	1		20.1	RAWSRLVLGSGNIYF	1.3
	WL1-T-E6	1		20.1	CAWDRLARFQETQYF	2.5
WL2	WL2-T-E47	1		1.1	CASSVTPGGTEAFF	1.1
	WL2-T-E39	1		1.1	CASSGTGEPTNEKLFF	1.4
	WL2-T-B69	2		2.1	CSADSGTSTDTQYF	2.3
	WL2-T-B55	1		3.1	CASSLNPELNYGYTF	1.2
	WL2-T-E41	1		3.1	CASGATGHHNSPLHF	1.6
	WL2-T-B63	1		3.1	CASRDSGTRNEQFF	2.1
	WL2-T-B71	1		3.1	CASGFGSGSLTDTQYF	2.3
	WL2-T-B76	4		3.1	CASSTTQGFYEQYF	2.7
	WL2-T-B68	1		5.4	CASSLGLAGDQETQYF	2.5
	WL2-T-E45	1		5.6	CASNNGLAHTPEYF	2.5
	WL2-T-B70	1		6.1	RASTTKGLRDSPLHF	1.6
	WL2-T-B61	4		6.1	CASSQPAGPSTDTQYF	2.3
	WL2-T-E36	1		6.4	CASSSYLWTGNLSPLHF	1.6

	WL2-T-E58	1		6.5	CASSLGQNEQYF	2.7
	WL2-T-B57	1		6.5	CASSLGQGEQYF	2.7
	WL2-T-B59	2		6.5	CASSFLGQFSYEQYF	2.7
	WL2-T-B75	1		6.5	CASSLDSDHEQYF	2.7
	WL2-T-B64	1		8.1	CASSLGGEVSGQGNQPQHF	1.5
	WL2-T-E37	1		11.1	CASSDARTGDIGQYF	2.3
	WL2-T-E53	1		11.1	CASSDPTGTGANVLTF	2.6
	WL2-T-E52[c]	2		11.1	CASSDAGTGDYEQYF	2.7
	WL2-T-B66	3		13.1	CASGPLGGDNQPQHF	1.5
	WL2-T-B73	2		13.3	CASSEIAGGLGFF	2.1
	WL2-T-B58	1		15.1	CATSDTGTSVGGQYF	2.5
	WL2-T-E46	1		21.1	CASSSSESGSYNEQFF	2.1
	WL2-T-E38	4		22.1	CASVPGLSYEQYF	2.7
SL	SL-G-A58		1	1.1	CASSVTPGHTEAFF	1.1
	SL-G-D55		1	1.1	CASSVAGGETEAFF	1.1
	SL-G-A45		4	1.1	CASSVAPSANTGELFF	2.2
	SL-G-D56		1	1.1	CASSFSPGPPEGELFF	2.2
	SL-G-A62		2	3.1	CASSLEEQRAFF	1.1
	SL-G-A47		5	3.1	CASNSGYMNFF	2.1
	SL-G-D54		2	11.1	CASSEDRRGSYEQYF	2.7
	SL-G-A54[c]		2	11.1	CASSDAGTGDYEQYF	2.7
	SL-G-A57		1	13.1	CASSPVQGASGNTIYF	1.3
	SL-G-A69		1	13.1	CASSYVDGSSYEQYF	2.7
	SL-G-A52		2	20.1	CAWSPLEIAGTDTQYF	2.3
	SL-G-A41	6	8	1.1	CASSVAPGSEGEAFF	1.1
	SL-G-A60	9	2	2.1	CSAGGWDRVNQPQHF	1.5
	SL-G-A40	16	5	11.1	CASSESQTGDYEQYF	2.7
	SL-G-A46	10	1	15.1	CATSDSTSGGTDTQYF	2.3
	SL-T-B45[d]	4		1.1	CASSFSAEATGELFF	2.2
	SL-T-B32	2		3.1	CASSRLEARELFF	2.2
	SL-T-B37	2		5.1	CASSLTNRGLNMNTEAFF	1.1
	SL-T-B51	1		5.1	CASSLTGGSETQYF	2.5
	SL-T-B33	1		6.1	CASSVPGEESGRGNTEAFF	1.1
	SL-T-C10	1		6.5	CASSLGRGEQYF	2.7
	SL-T-C9	1		7.2	CASSRGTENEKLFF	1.4
	SL-T-B43	1		10.1	CASSKKLDSFSYEQYF	2.7
	SL-T-E20	1		11.1	CASSGSGPLRGYTF	1.2
	SL-T-E15	1		11.1	CASSPAGPGNTIYF	1.3
	SL-T-C19	3		11.1	CASSDPGHQNSPLHF	1.6
	SL-T-E16[d]	4		11.1	CASSFSAEATGELFF	2.2
	SL-T-B34	1		13.1	CASSYEGTEAFF	1.1
	SL-T-E28	1		13.1	CASSYDLEPTNEKLFF	1.4
	SL-T-B48	1		13.1	CASSYSGSLGEKLFF	1.4
	SL-T-E19	1		13.1	CASIEQGALTNEQFF	2.1
	SL-T-B38	2		13.1	CASTLDRDGTQYF	2.5
	SL-T-C22	1		13.1	CASSGSPGQGAEYF	2.7
	SL-T-E30	1		15.1	CATSAGGAEPTDTQYF	2.3
	SL-T-E32	2		23.1	CASSWRGGIQGVAGELFF	2.2

[a] Vβ nomenclature adapted from Arden, et al, 1995.

[b] Acidic residues in CDR3 are shown in red and basic residues are shown in blue.

[c] WL2-T-E52 and SL-G-A54 have the same Protein translation but a silent mutation in CDR3

[d] SL-T-B45 and SL-T-E16 have the same CDR3, different Vβ.

Table IV.2. *Previously published V β TCRs specific for DR-Ha* [a]

Clone	Restriction	Vβ[b]	CDR3[c]	Jβ	Reference
3BC6.6	B1*0401	3.1	CASSLTGTGYTF	1.2	Brawley 96
JS515	B1*0701	3.1	CASSPGTSGTTYEQYF	2.7	Brawley 96
HA1.7	B1*0101	3.1	CASSSTGLPYGYTF	1.2	Hewitt 92
HAR	B1*1101	3.1	CASRVIDSTEAFF	1.1	Ostrov 93
GS34	B1*0401	3.1	CASRPSPGSQVRGVFF	1.4	Prevost-Blondel 95
GS6	B1*0401	3.1	CASNSIAGGPSYEQYF	2.7	Prevost-Blondel 95
GS24	B1*1101	3.1	CASSSSYGYTF	1.2	Prevost-Blondel 95
AND.21	B1*1101	3.1	CASSYQEANTGELFF	2.2	Prevost-Blondel 95
AND.7	B1*1101	3.1	CASTPGQETQYF	2.5	Prevost-Blondel 95
AND.10	B1*1101	3.1	CASSLQGYEQYF	2.7	Prevost-Blondel 95
GS11	B1*1101	15.1	CATSEPDRXYEQFF	2.1	Prevost-Blondel 95
28	B1*0401	3.1	CASAGGGRGNEKLFF	1.4	Snoke 93
14	B1*0401	3.1	CASSIDGPQHF	1.5	Snoke 93
29	B1*0401	3.1	CASSSSGRAPEQFF	2.1	Snoke 93
Cl-1	B1*0101	13.1	CASRDFLSGEQYF	2.7	Wedderburn 95
HC4-1	B1*0101	3.1	CASSGSGPIYHSPLHF	1.6	Yassine-Diab 99
B103	B1*0101	5.2	CASSLGLAGGQDTQYF	2.3	Yassine-Diab 99
NF4	B1*0101	8.2	CASSFWTGPRADTQYF	2.3	Yassine-Diab 99
B151	B1*0101	9.1	CASSEVPGQRINSPLHF	1.6	Yassine-Diab 99
B105	B1*0101	11.1	CASSDGTFTEAFF	1.1	Yassine-Diab 99
NF3	B1*0101	11.1	CASSESGRHRETQYF	2.5	Yassine-Diab 99
HC6	B1*0101	11.1	CASSESGTGDYEQYF	2.7	Yassine-Diab 99
NF2	B1*0101	13.1	CASSYQPGTEGKLFF	1.4	Yassine-Diab 99
NF1	B1*0101	15.1	CTSGWTSGSTGELFF	2.2	Yassine-Diab 99
BH10	B1*0103	3.1	CASSEPITGSRDGYTF	1.2	Yassine-Diab 99
BC24	B1*0103	3.1	CASSFNRIFGNTIYF	1.3	Yassine-Diab 99
B08	B1*0103	3.1	CASSFPKTAGAYEQYF	2.7	Yassine-Diab 99
BC16	B1*0103	3.1	CASSFPRTGGKTLVHSYEQYF	2.7	Yassine-Diab 99
BC38-1	B1*0103	11.1	CASSEYKNSFPGEQYF	2.7	Yassine-Diab 99
B11	B1*0103	14.1	CASSPRLGPGEKLFF	1.4	Yassine-Diab 99
B09	B1*0103	20.1	CAWSTPTGTRDTQYF	2.3	Yassine-Diab 99
BC01	B1*0103	20.1	CAWGKGSGANVLTF	2.6	Yassine-Diab 99

[a] Table based on data originally compiled in Hennecke, et al., 2000.

[b] Vβ nomenclature adapted from Arden, et al, 1995.

[c] Acidic residues in CDR3 are shown in red and basic residues are shown in blue.

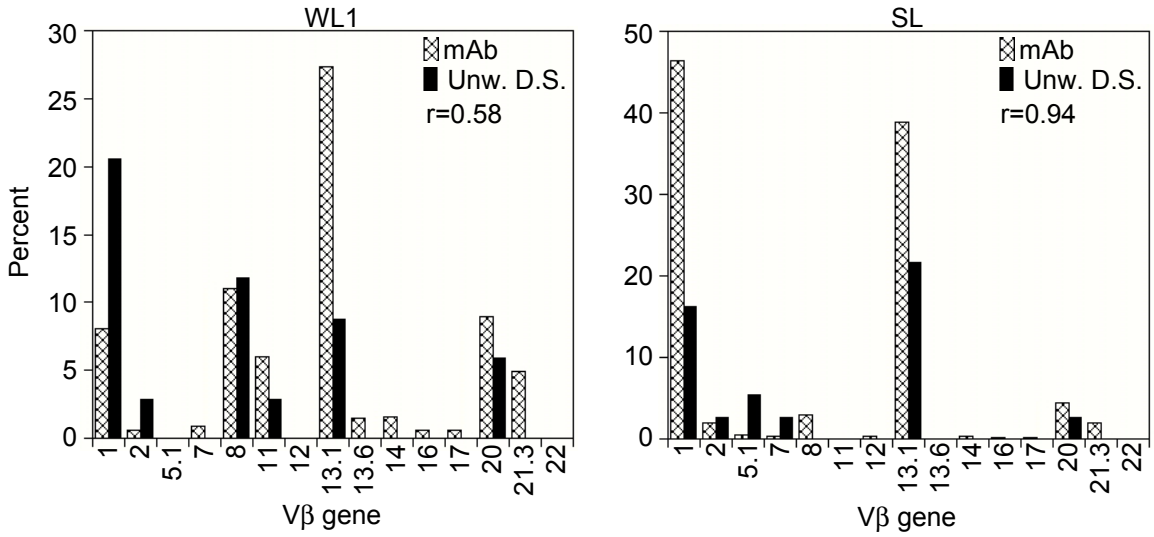


Figure IV.5. *Vβ usage assessed by mAb analysis and direct sequencing are similar.* Percent of DR1-Ha oligomer staining cells which co-stained with particular anti-Vb mAbs are plotted (hatched bars) next to percent of clones identified by the direct sequencing approach (closed bars) for individuals WL1 (a) and SL (b). The sequences were analyzed without weighting by number of occurances (unweighted). Correlation coefficients (r) for the data sets are indicated. Data from mAb was collected on the cells two weeks after the same pools were analyzed by direct sequencing.

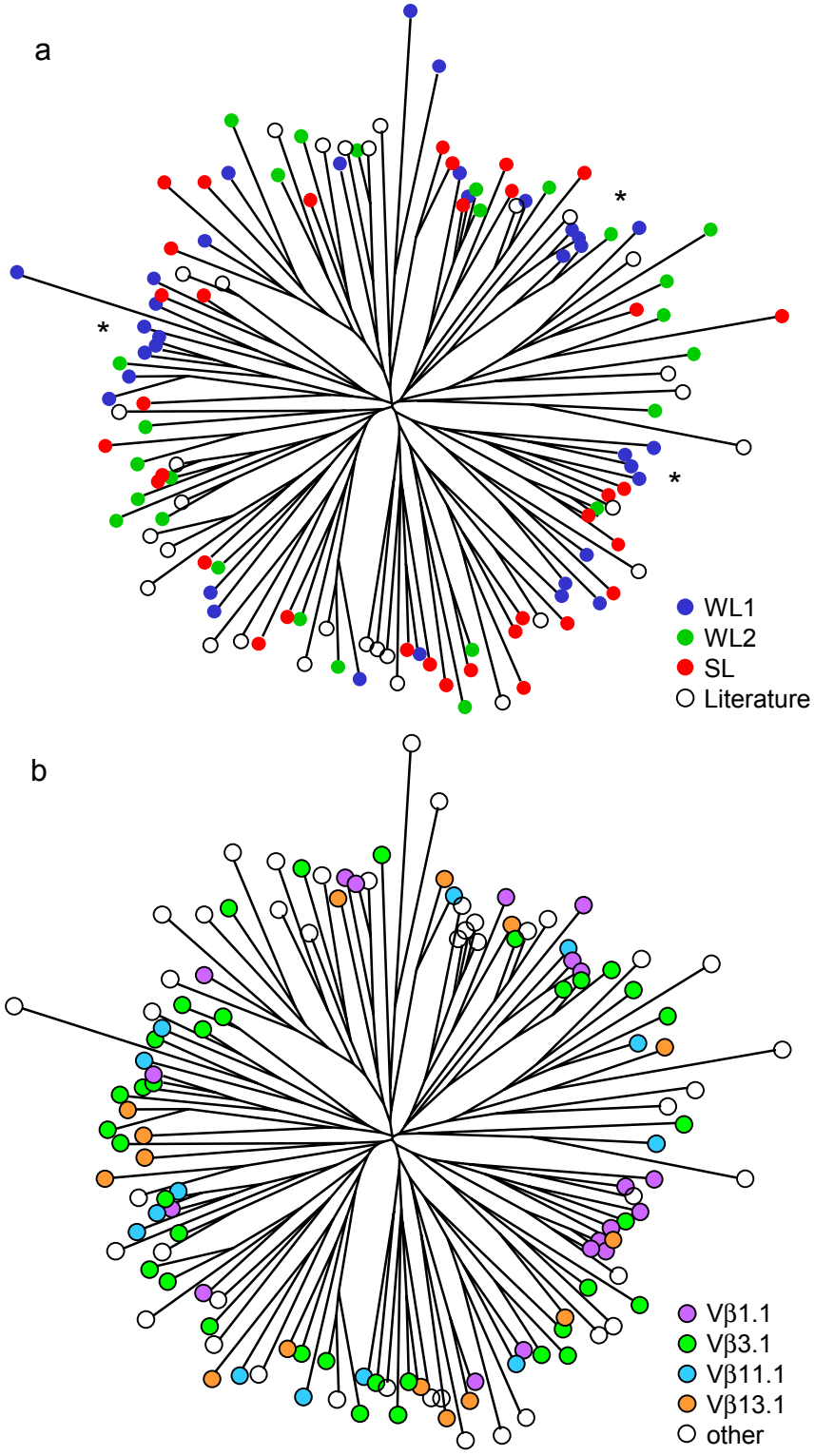


Figure IV.6. *CDR3 similarity ees.* The CDR3 regions from TCRβ sequences in Table I and Table II were scored for similarity using the ClustalW algorithm and mapped using eeView software. Sequences were color coded for either sequence source (a) or associated Vβ (b).

Table IV.3. *Convergent Evolution of TCR β CDR3 region*

ID/Clone	Vβ	CDR3[a]	Jβ
HA1.7	3.1	CASSS**TGLP**YGYTF	1.2
GS24	3.1	CASSS**S**---YGYTF	1.2
SL-T-B32	3.1	CASS**RL**EA**R**--ELFF	2.2
AND.21	3.1	CASS**YQ**EA**NTG**ELFF	2.2
SL-T-B45	1.1	CASSFSAEATGELFF	2.2
SL-T-E16	11.1	CASSFSAEATGELFF	2.2
SL-T-E15	11.1	CASSP**AG**--**P**GNTIYF	1.3
SL-G-A57	13.1	CASSP**VQGA**SGNTIYF	1.3
WL1-G-A1	8.1	CASS**LSQ**G**DQ**PQHF	1.5
14	3.1	CASS**ID**-G--PQHF	1.5
SL-T-B38	13.1	CASTL**D**R**D**G--**T**QYF	2.5
SL-T-C10	6.5	CASS**LGR**-G--**EQY**F	2.7
WL1-G-A13	6.5	CASS**V**G**Q**-G--**KAF**F	1.1
WL1-G-A18	6.5	CASS**L**GQ-**G**--**EQF**F	2.1
WL2-T-E58	6.5	CASSLGQ-**N**--EQ**Y**F	2.7
WL2-T-B57	6.5	CASSL**G**Q-**G**--EQYF	2.7
AND.10	3.1	CASSL-Q-G-**Y**EQYF	2.7
WL2-T-B76	3.1	CASST**T**Q-G**FY**EQYF	2.7
WL1-T-D3	3.1	CASSL**V**PEL**GE**QYF	2.7
WL1-G-D41	3.1	CASSL**S**PEL**ET**QYF	2.5
WL1-T-B18	3.1	CASSL**A**PEL**D**TQYF	2.3
WL2-T-B63	3.1	CAS**R**DSG--T**R**NEQFF	2.1
WL1-T-B16	3.1	CAS**SDS**G--T**Y**NEQFF	2.1
WL1-T-B2	3.1	CASS**PG**G**GD**TYNEQFF	2.1
B105	11	CASS**D**G**T**F**T**E**A**FF	1.1
WL1-T-B22	1.1	CASS**T**G-F**S**E**Q**FF	2.1
WL1-T-D22	1.1	CASS**A**G-FSEQFF	2.1
WL1-T-D17	13.1	CASS**RE**GT**VNHG**GYTF	1.2
3BC6.6	3.1	CASS**LT**GT----GYTF	1.2

ID/Clone	Vβ	CDR3[a]	Jβ
WL1-T-E10	8.1	CASS**SD**G**T**-GEKLFF	1.4
SL-T-B48	13.1	CASS**YS**G**SL**GEKLFF	1.4
B103	5.2	CASSLGLAG**G**Q**D**TQYF	2.3
WL2-T-B68	5.4	CASS**LGLAGD**Q**E**TQYF	2.5
AND.7	3.1	CAST**P**----**G**QETQYF	2.5
WL1-G-A10	3.1	CAS**TQGVL**--**T**YEQYF	2.7
WL2-T-E38	22.1	CAS**V**PG**L**S---YEQYF	2.7
JS515	3.1	CAS**S**PG**T**S**GTT**YEQYF	2.7
WL2-T-E39	1.1	CASS**GTG**EPTNEKLFF	1.4
SL-T-E28	13.1	CASS**YDL**EPTNEKLFF	1.4
WL1-G-A35	1.1	CASSV**G**P**G**P**GD**TEAFF	1.1
WL1-T-D39	1.1	CASSVA**PS**P**S**ETEAFF	1.1
SL-G-D55	1.1	CASSV**AG**G--**E**TEAFF	1.1
WL2-T-E47	1.1	CASSV**TP**G--**G**TEAFF	1.1
SL-G-A58	1.1	CASSVTPG--**H**TEAFF	1.1
SL-G-A40	11.1	CASSES**Q**TGDYEQYF	2.7
HC6	11	CASS**ESG**TGDYEQYF	2.7
WL2-T-E52	11.1	CASS**DA**GTGDYEQYF	2.7
SL-G-A54	11.1	CASSDAGTGDYEQYF	2.7
WL2-T-B68	5.4	CASSLGLAG**D**Q**E**TQYF	2.5
B103	5.2	CASSLGLAG**G**Q**D**TQYF	2.3
BC16	3.1	CASSFP**R**T**G**G**KTLVHS**YEQYF	2.7
B08	3.1	CASSFP**K**T**A**G-----**A**YEQYF	2.7
WL2-T-E39	1.1	CASS**GTG**EPTNEKLFF	1.4
SL-T-E28	13.1	CASS**YDL**EPTNEKLFF	1.4
WL1-G-A35	1.1	CASSV**G**P**G**P**GD**TEAFF	1.1
WL1-T-D39	1.1	CASSV**A**P**S**P**SE**TEAFF	1.1

Table IV.4. *Statistical Comparison of Vβ usage in Validated and Non-validated TCR β sequen*

Pool	# of Seq in both Tet & IFN-γ	# of Seq related to another	# of Seq Validated[a]	# of Seq Non-validated[b]	Vβ Usage Comparison[c] r	p	χ^2	DF	N
Tet	13	31	39	42	0.61	0.62	26.2	29	79
IFN-γ	13	11	19	11	0.75	1.00	7.0	29	30
Both	13	37	45	50	0.74	0.69	24.8	29	96

[a] TCRβ sequences were validated if they were either detected in both the IFN-γ and the Tet pools, or if they were related to another TCRβ sequence either newly identified (Table I) or previously published in the literature (Table II).

[b] TCRβ sequences were considered suspect if they were not validated.

[c] Statistical parameters were determined by comparing the Vβ usage distributions of the validated and suspect TCRβ sets.

Table IV.5. TCR *V β gene family usage*

	Percent of Population[a]										
	Weighted				Unweighted				all	Lit	IMGT
Vβ	WL1	WL2	SL	All	WL1	WL2	SL	All	Lit[b]	*0101[c]	Nomenclature[d]
1.1	**12.5**	4.8	**19.8**	**14.3**	**20.6**	7.4	**16.2**	**15.3**	0.0	0.0	TRBV9
2.1	8.3	4.8	9.9	8.4	2.9	3.7	2.7	3.1	0.0	0.0	TRBV20-1
3.1	**27.5**	**19.0**	8.1	**18.3**	**38.2**	**18.5**	8.1	**21.4**	**57.6**	**18.2**	TRBV28
5.1	0.0	0.0	2.7	1.1	0.0	0.0	5.4	2.0	0.0	0.0	TRBV5-1
5.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.0	9.1	TRBV5-6
5.4	0.0	2.4	0.0	0.4	0.0	3.7	0.0	1.0	0.0	0.0	TRBV5-8
5.6	0.0	2.4	0.0	0.4	0.0	3.7	0.0	1.0	0.0	0.0	TRBV5-4
6.1	0.0	11.9	0.9	2.2	0.0	7.4	2.7	3.1	0.0	0.0	TRBV7-3
6.4	0.0	2.4	0.0	0.4	0.0	3.7	0.0	1.0	0.0	0.0	TRBV7-9
6.5	**17.5**	11.9	0.9	9.9	5.9	**14.8**	2.7	7.1	0.0	0.0	TRBV7-2
6.6	0.8	0.0	0.0	0.4	2.9	0.0	0.0	1.0	0.0	0.0	TRBV7-7
7.2	0.0	0.0	0.9	0.4	0.0	0.0	2.7	1.0	0.0	0.0	TRBV4-3
8.1	**19.2**	2.4	0.0	8.8	**11.8**	3.7	0.0	5.1	0.0	0.0	TRBV12-3
8.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.0	9.1	TRBV12-4
9.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.0	9.1	TRBV3-1
11.1	5.8	9.5	**29.7**	**16.1**	2.9	**11.1**	**18.9**	**11.2**	**12.1**	**27.3**	TRBV25-1
13.1	6.7	9.5	8.1	7.7	8.8	3.7	**21.6**	**12.2**	6.1	**18.2**	TRBV6-5
13.3	0.0	2.4	0.0	0.4	0.0	3.7	0.0	1.0	0.0	0.0	TRBV6-1
14.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.0	0.0	TRBV27
15.1	0.0	2.4	10.8	4.8	0.0	3.7	5.4	3.1	6.1	9.1	TRBV24-1
20.1	1.7	0.0	1.8	1.5	5.9	0.0	2.7	3.1	6.1	0.0	TRBV30
21.1	0.0	2.4	0.0	0.4	0.0	3.7	0.0	1.0	0.0	0.0	TRBV11-1
22.1	0.0	9.5	0.0	1.5	0.0	3.7	0.0	1.0	0.0	0.0	TRBV2
23.1	0.0	0.0	1.8	0.7	0.0	0.0	2.7	1.0	0.0	0.0	TRBV13

Unrepresented functional TCRVβs:

Vβ	IMGT	Vβ	IMGT	Vβ	IMGT	Vβ	IMGT
4.1	TRBV29-1	7.3	TRBV4-2	13.2b	TRBV6-3	17.1	TRBV19
5.3	TRBV5-5	8.3	TRBV12-5	13.4	TRBV6-9	18.1	TRBV18
6.2	TRBV7-8	12.1	TRBV10-3	13.5	TRBV6-4	21.2	TRBV11-3
6.3	TRBV7-6	12.2	TRBV10-1	13.6	TRBV6-6	21.3	TRBV11-2
6.8	TRBV7-4	12.3	TRBV10-2	13.7	TRBV6-8	24.1	TRBV15
7.1	TRBV4-1	13.2a	TRBV6-2	16.1	TRBV14	25.1	TRBV16

[a] Data points with $p < 0.05$ (from chi-squared analysis comparing to random distribution among all 48 functional Vβs) are indicated in bold.

[b] "All Lit" includes previously published TCRβ sequences known to be specific for Ha peptide bound to any HLA-DR allele.

[c] "Lit *0101" includes only B1*0101-restricted Ha-specific TCRβ sequences.

[d] We have included the Vb nomenclature of the International Immunogenetics Database (IMGT) (Lefranc, et al REFXXX) in this table because it has been recently approved by the Human Genome Organization Nomenclature Committee (REFXXX) and we believe that it

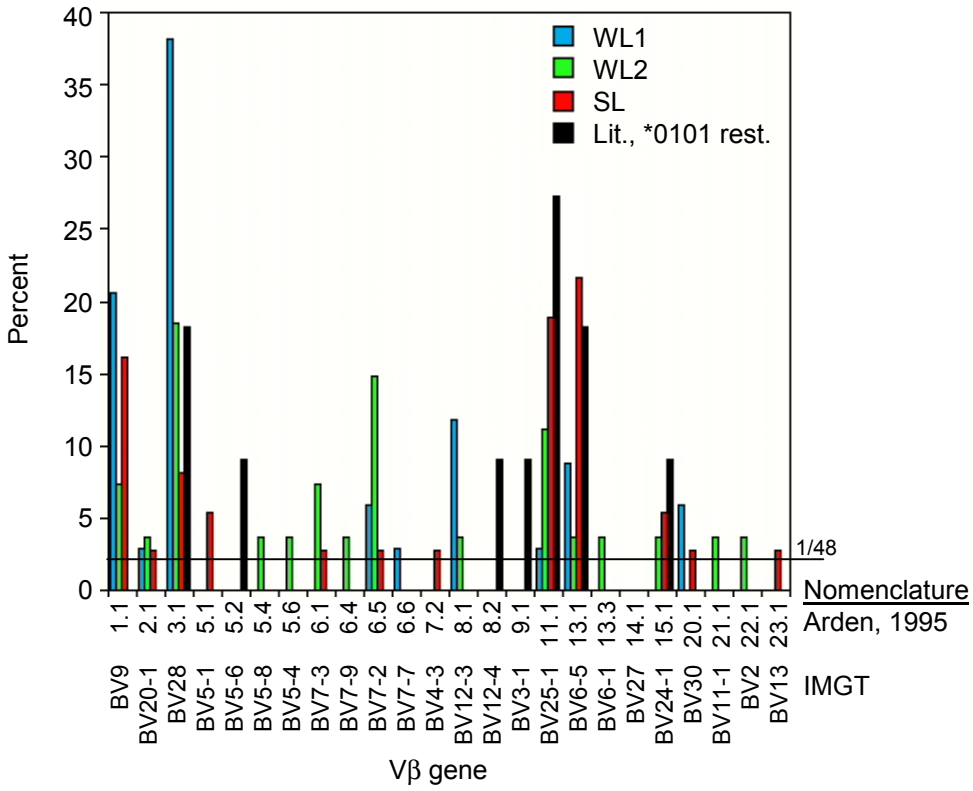


Figure IV.7. *Vβ usage of DR1-Ha specific TCRβ sequences.* Percent of sequences (unweighted by number of occurances) with particular Vβs are plotted for individuals WL1 (blue), WL2 (green), SL (red), and for the DR1-resicted clones from the literature (black). Vβ are described by both the nomenclatures of Arden, 1995 and IMGT. Random occurance of Vβ genes (1 in 48 functional genes) is indicated.

Table IV.6. *Comparison of Vβ usage by different individuals*

Weighted				Unweighted		
WL1	WL2	SL	Vβ	WL1	WL2	SL
15	2	22	1.1	7	2	6
10	2	11	2.1	1	1	1
33	8	9	3.1	13	5	3
0	0	3	5.1	0	0	2
0	1	0	5.4	0	1	0
0	1	0	5.6	0	1	0
0	5	1	6.1	0	2	1
0	1	0	6.4	0	1	0
21	5	1	6.5	2	4	1
1	0	0	6.6	1	0	0
0	0	1	7.2	0	0	1
23	1	0	8.1	4	1	0
7	4	33	11.1	1	3	7
8	4	9	13.1	3	1	8
0	1	0	13.3	0	1	0
0	1	12	15.1	0	1	2
2	0	2	20.1	2	0	1
0	1	0	21.1	0	1	0
0	4	0	22.1	0	1	0
0	0	2	23.1	0	0	1

r_{12}	0.68
r_{2S}	0.40
r_{1S}	0.34
p	0.00
χ^2	185.9
DF	58
N	270

r_{12}	0.69
r_{2S}	0.43
r_{1S}	0.45
p	0.40
χ^2	60.2
DF	58
N	95

Table IV.7. *Comparison of Vβ usage by patients and literature*

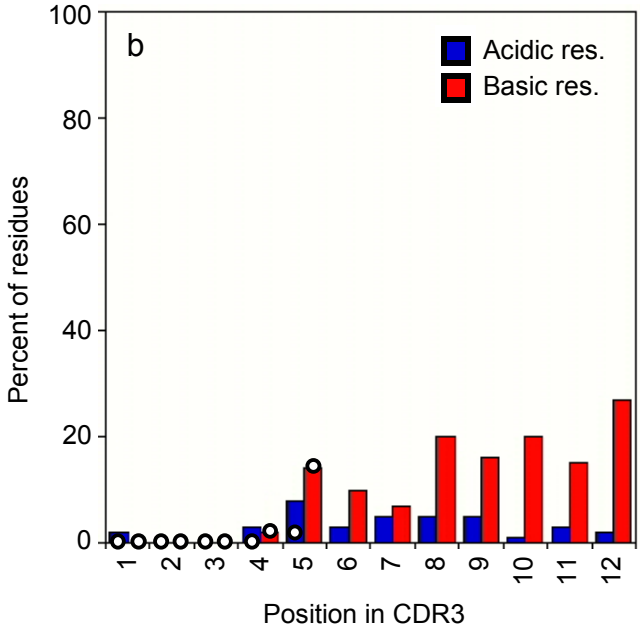
Weighted			Unweighted	
New	Lit	Vβ	New	Lit
39	0	1.1	15	0
23	0	2.1	3	0
50	19	3.1	21	19
3	0	5.1	2	0
0	1	5.2	0	1
1	0	5.4	1	0
1	0	5.6	1	0
6	0	6.1	3	0
1	0	6.4	1	0
27	0	6.5	7	0
1	0	6.6	1	0
1	0	7.2	1	0
24	0	8.1	5	0
0	1	8.2	0	1
0	1	9.1	0	1
44	4	11.1	11	4
21	2	13.1	12	2
1	0	13.3	1	0
0	1	14.1	0	1
13	2	15.1	3	2
4	2	20.1	3	2
1	0	21.1	1	0
4	0	22.1	1	0
2	0	23.1	1	0

r	0.61
p	0.01
χ^2	73.8
DF	29
N	303

r	0.72
p	0.09
χ^2	39.6
DF	29
N	128

a

	CDR1		CDR2		CDR3	
	Net Crg	# Crgs	Net Crg	# Crgs	Net Crg	# Crgs
IFN-γ	-0.9	3.0	-0.6	3.9	-1.4	2.0
Tet	-1.0	2.7	-0.9	3.6	-1.0	1.9
Lit	-2.6	3.5	-0.6	4.8	-0.4	1.8
All 48 Vβ[b]	-0.1	1.9	-1.2	3.6		



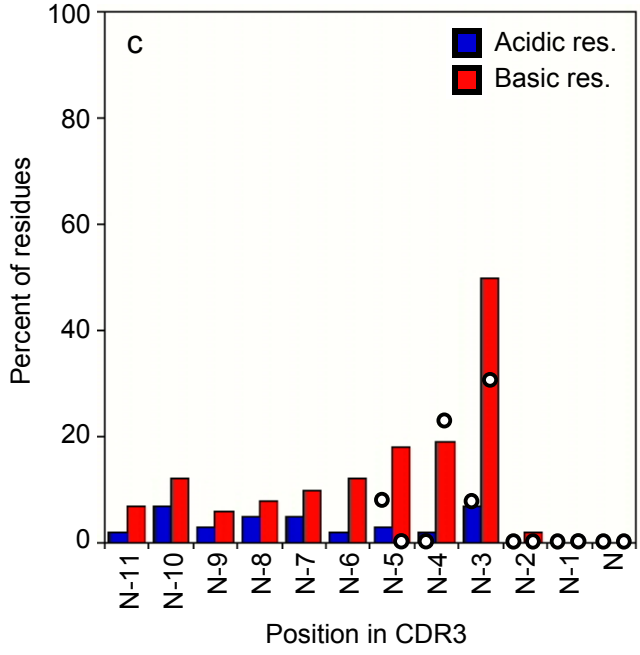


Figure IV.8. *Analysis of charged residues in CDR regions.* (a) CDR regions from TCRb sequences in Table I were analyzed for average charge characteristics in CDR1, CDR2 and CDR3. Average net charge and average number of charges is reported. (b,c) CDR3 regions of TCRβ sequences listed in Table I were aligned at either their N-terminal Cys residue (b, Pos. 1) or C-terminal Phe residue (c, Pos. N). Percent of acidic (blue) and basic (red) residues at each of twelve positions relative to those starting points are plotted. Frequency of acidic and basic residues in all 48 Vβ (b) or in all 13 Jβ (c) segments are indicated with open circles for the CDR3 positions at which they could have potentially contributed. HA1.7 contacts DR1-Ha at positions 5, 6, 7, and 9, or N09, N-8, N-7 and N-5. The CDR3 of HA1.7 TCRβ contains no charged residues.

Table IV.8. *Comparison of Tet and IFN-γ pools*

Analysis by Vβ usage[a]

WL1

Wghtd Tet	Wghtd IFN-γ	Vβ	Unwghtd Tet	Unwghtd IFN-γ
12	3	1.1	6	3
10	0	2.1	1	0
19	14	3.1	10	6
8	13	6.5	2	2
1	0	6.6	1	0
8	15	8.1	2	3
7	0	11.1	1	0
8	0	13.1	3	0

r	0.70	r	0.92
p	0.39	p	1.00
χ^2	30.6	χ^2	5.1
DF	29	DF	29
N	120	N	40

SL

Wghtd Tet	Wghtd IFN-γ	Vβ	Unwghtd Tet	Unwghtd IFN-γ
10	15	1.1	2	5
9	2	2.1	1	1
2	7	3.1	1	2
3	0	5.1	2	0
1	0	6.1	1	0
1	0	6.5	1	0
1	0	7.2	1	0
25	9	11.1	5	3
7	2	13.1	6	2
11	1	15.1	2	1
0	2	20.1	0	1
2	0	23.1	1	0

r	0.66	r	0.64
p	0.45	p	1.00
χ^2	29.3	χ^2	10.3
DF	29	DF	29
N	111	N	41

Analysis by Individual TCRβ sequences[b]

WL1, Tet v. IFN-γ

Wghtd		Unwghtd	
r	0.40	r	-0.55
p	0.00	p	0.87
χ^2	60.5	χ^2	24.0
DF	33	DF	33
N	120	N	40

SL, Tet v. IFN-γ

Wghtd		Unwghtd	
r	0.36	r	-0.75
p	0.00	p	0.69
χ^2	68.9	χ^2	31.4
DF	36	DF	36
N	111	N	41

[a] Distribution of Vβ usage in Tet and IFN-γ pools within individuals is shown with or without weighting by the number of occurances of particular TCRβs. Statistical parameters were determined as described in Methods.

[b] Distribution of TCRβ sequences within Tet and IFN-γ pools of each individual are shown in Table I. Statistical parameters were determined by comparing Tet and IFN-γ pools within each individual, either with or without weighting, as described in Method

V. Investigation of the T cell activation trigger

V. A. Current understanding of T cell activation

The mechanism of T cell activation remains an important unsolved problem. Interaction of T cell receptor with cognate MHC-peptide ligands on an antigen-presenting cell is well established to be the critical event determining the specificity of T cell activation. However, many different interactions are involved in initiation and modulation of the character and extent of T cell activation. Some of the most important protein components of T cell activation are summarized in Table V.1. Despite having identified many of the key proteins involved, the transmembrane signaling mechanism and subsequent order of membrane-proximal activation events in the cytoplasm is still poorly understood. In addition to these protein-based interactions, it is now clear that membrane organization is another important mediator of T cell activation. Membrane microdomains, commonly termed lipid rafts, have been shown to be enriched in T cell signaling molecules after TCR engagement (Viola et al 1999). Large-scale molecular reorganization and segregation has been observed to correlate with productive T cell activation (Grakoui et al 1999; Monks et al 1998). And the reversible membrane binding of the cytoplasmic tail of TCRζ has been proposed to mediate signaling (Aivazian & Stern 2000). Currently, there appear to be too many partially understood variables in the activation of T cells to allow researchers to piece the puzzle together.

In this chapter I will briefly summarize some of the work that I have been involved in probing the triggering mechanism of T cell receptor on $CD4^+$ T cells. I will finish this chapter with a short discussion of a novel mechanism of T cell activation that we recently proposed.

V. B. Experimental investigation of T cell triggering mechanism

V. B.1 Transmembrane signal transduction models

We chose to simplify the problem of T cell activation by examining only the antigen-specific reaction, TCR binding to MHC-peptide, and trying to determine the key parameters regulating TCR activation, what we termed TCR triggering. For this triggering event we hypothesized three models of transmembrane signaling (Fig. V.1) based on other membrane receptor signaling systems. In the first mechanism (Fig. V.1a), typical of seven transmembrane span receptors that activate G-proteins (Wess et al 1997), ligand binding induces a structural change in the receptor that can be sensed by effector proteins within the cell. In another mechanism (Fig. V.1b), binding of a multivalent ligand induces receptor co-localization. This mechanism is typical of receptor tyrosine kinases, in which receptor clustering facilitates transphosphorylation by the cytoplasmic kinase domains (Hubbard & Till 2000). In a third mechanism (Fig. V.1c), ligand binding induces a rearrangement of a receptor oligomer into a specific activating conformation. One example of this mechanism is the bacterial aspartic acid receptor, in which ligand binding induces a helix reorganization that activates receptor-associated cytoplasmic signaling proteins (Falke et al 1997). We have recently reviewed these mechanisms and research approaches using various oligomeric MHC-based reagents (Cochran et al 2001a).

V. B.2 TCR dimerization is the critical determinant of T cell triggering

We investigated the T cell triggering mechanism by replacing the antigen-presenting cell with soluble oligomers of MHC-peptide complexes. This work was primarily performed

by Jennifer Cochran in our lab (Cochran et al 2000; Cochran et al 2001b), with assistance from myself and, later, Jennifer Stone.

We formed soluble oligomers of MHC-peptide complexes and tested them as T cell activators, as measured by down-regulation of TCR and upregulation of the cell surface markers CD25 and CD69. Using Ha-specific, DR1-restricted $CD4^+$ T cell clones and lines we showed that DR1-peptide monomers bound to TCR on these cells but failed to trigger them. Monomer, dimers, trimers and higher oligomers were each able to trigger activation, with potency increasing with oligomer valency. However, when oligomer binding was measured simultaneously with the T cell activation markers, each oligomer species was shown to trigger equivalent extent of activation on a per-MHC-bound basis. These results made clear that TCR dimerization was the critical event in triggering the earliest activation events of $CD4^+$ T cells (Cochran et al 2000). Because monomeric engagement of TCR failed to activate, these results rule out allosteric change in receptor conformation (Fig. V.1a) as a triggering mechanism for $CD4^+$ T cells.

V. B.3 Receptor proximity, not receptor orientation, determines T cell triggering

The triggering mechanism was further examined using an array of topologically distinct MHC-peptide dimers. Dimers in constrained topologies, which necessarily cluster TCR in different relative orientations, were nonetheless similarly capable of triggering $CD4^+$ T cell activation. However, dimers constructed with longer crosslinkers were significantly less potent than dimers constructed with shorter crosslinkers. These results show that receptor proximity, and not receptor orientation, is critical for triggering activation through TCR (Cochran et al

2001b). These results argue against a ligand induced receptor rearrangement model (Fig. V.1c) and favor receptor co-localization (Fig. V.1b) as the critical mediator of T cell triggering.

V. C. TCR losing its inhibitions: a novel mechanism of TCR triggering

Recently, Reth reviewed data suggesting that the antigen receptors of both B cells and T cells are present in oligomeric forms on the surface of resting cells, and proposed new models for antigen receptor transmembrane signaling (Reth 2001). In his proposed model for B cell triggering, the BCR has a defined oligomeric structure that in the absence of antigen leads to a maintenance signal; disturbance of the oligomeric structure by antigen binding leads to an activation signal. A similar model was proposed for T cell triggering. While the T cell model is inconsistent with experiments demonstrating that $CD4^+$ T cells are not activated by monovalent engagement of their antigen receptors, a variant of the model can accommodate these data. We described this model recently in a letter to Trends in Immunology (Cameron et al 2001). The revised model has interesting implications for possible T cell triggering mechanisms.

In Reth's proposed model (Fig. V.2a), the resting TCR is an inert oligomer. Binding of a MHC-peptide complex to a single TCR induces a conformational change, resulting in exposure of signaling motifs within the cytoplasmic domains of that receptor. Binding of another MHC-peptide complex to a different TCR within the same oligomer would cause cross-phosphorylation of associated kinases and signal amplification. Weakly-binding or short-lived peptide complexes not able to induce signaling on their own could be active in the context of an oligomer already engaged by activating peptides.

While this model is consistent with observations of TCR oligomers on resting cells (Fernandez-Miguel et al 1999), it is inconsistent with studies demonstrating a requirement for multivalent engagement of TCR for initiation of signaling. For $CD4^+$ T cells, soluble monomeric MHC-peptide complexes are unable initiate signaling (Boniface et al 1998; Cochran & Stern 2000), even under conditions where they engage the majority of cell-surface TCR (Cochran & Stern 2000). Indeed, we have observed that MHC-peptide monomers can block the activation induced by soluble oligomers (unpublished observations). Furthermore, MHC-TCR crystal structures have revealed no significant structural changes induced by binding that could be propagated into the T cell (Hennecke & Wiley 2001), as would be required by a model in which a single occupied TCR could initiate signaling.

A triggering model based on TCR cross-linking (Fig. V.1b) has long been favored in the field, primarily stimulated by experiments demonstrating that T cells can be activated by antibody-mediated cross-linking of TCR or its associated CD3 subunits, or even by cross-linking of chimeric proteins carrying TCR cytoplasmic domains. In a current version of this model, TCR clustering initiates signaling processes by some combination of transactivation of associated kinases (Germain & Stefanova 1999), localization of engaged receptors to microenvironments that favor signaling (van der Merwe et al 2000), and conformational changes in receptor cytoplasmic domains caused by steric crowding or altered lipid environment (Aivazian & Stern 2000). TCR clustering could result from passive accumulation at the APC-T cell interface (Qi et al 2001), or could result from an active process involving costimulation (Germain & Stefanova 1999; van der Merwe et al 2000). However, it is not clear how any triggering model based on TCR crosslinking can be reconciled with observations of TCR already present as a preformed oligomer before activation.

It has been argued that much of the data used to support a cross-linking model are consistent also with a TCR triggering mechanism in which multivalent binding induces an allosteric change in a pre-existing receptor oligomer (Cochran et al 2001a; Reth 2001). Since there does not appear to be any dependence on the orientation of TCR in the activating state (Cochran et al 2001b), we have argued against conversion of an existing TCR oligomer into a specific activation arrangement (Fig. V.2b) as a key component of the activation signal (Cochran et al 2001a). However, in his model for BCR activation, Reth suggested that the resting oligomeric receptor might be in a specific inactivating arrangement, any disruption of which triggers activation (Reth 2001). It is possible to construct a model of T cell activation based on this idea (Figure V.2c). In this model, an inactive TCR oligomer constrains its MHC binding sites in a specific orientation inconsistent with multivalent engagement. Engagement by a multivalent ligand would disrupt this arrangement, resulting in changes to the environment of TCR transmembrane and/or cytoplasmic domains conducive to signaling. This model improves upon Reth's original TCR model by explaining the inability of soluble monomeric MHC-peptide ligands to activate $CD4^+$ T cells, and it does not require a ligand-induced TCR conformational change. Activation by anti-TCR antibodies and by immobilized MHC-peptide complexes could be accommodated by the model if these interactions also disrupted the key inhibitory TCR oligomer.

Table V.1 – Components of the T cell activation pathway

protein(s)	Function(s)
MHC proteins	Peptide binding proteins displayed on the surface of antigen presenting cells: Class I MHC proteins present peptides to $CD8^+$ cytotoxic T cells, Class II MHC proteins present peptides to $CD4^+$ helper T cells.
T cell receptor (TCR)	Multi-subunit, cell-surface complex; the αβ module carries hypervariable extracellular antigen binding domains that engage MHC-peptide complexes, and the non-variable CD3 δε, γε and ζζ modules carry cytoplasmic tyrosine-based signaling motifs (ITAMs).
CD4, CD8	T cell surface co-receptors for MHC molecules: CD4 binds class II MHC proteins, CD8 binds class I MHC proteins.
CD28, CTLA-4 / B7-1, B7-2	Co-stimulatory molecules found on surface of T cells / antigen presenting cells, which deliver activation signals in addition to those from TCR / MHC interaction, and may play a role in cytoskeletal rearrangements.
LFA-1 / ICAM CD2 / CD48	Adhesion molecules found on the surface of T cells / antigen presenting cells, which are important in mediating cell-cell contact and in formation of large-scale supramolecular clusters.
lck, fyn	Membrane-associated src-family tyrosine kinases responsible for initial phosphorylation of TCR ITAMs upon receptor engagement.
ZAP-70	Cytoplasmic, syk-family tyrosine kinases activated by binding to phosphorylated ITAMs.
CD45	Broad-spectrum protein tyrosine phosphatase that regulates lck activity and may play a role in regulating phosphorylation of TCR subunits.
vav, LAT, SLP-76	Cytoplasmic or membrane-associated adapter molecules carrying SH2, SH3, phosphotyrosine, and/or proline-rich domains, that bind to activated TCR components and engage downstream effector enzymes.
Roles for these many of these molecules in T cell activation have been reviewed recently (Dustin & Chan 2000).	

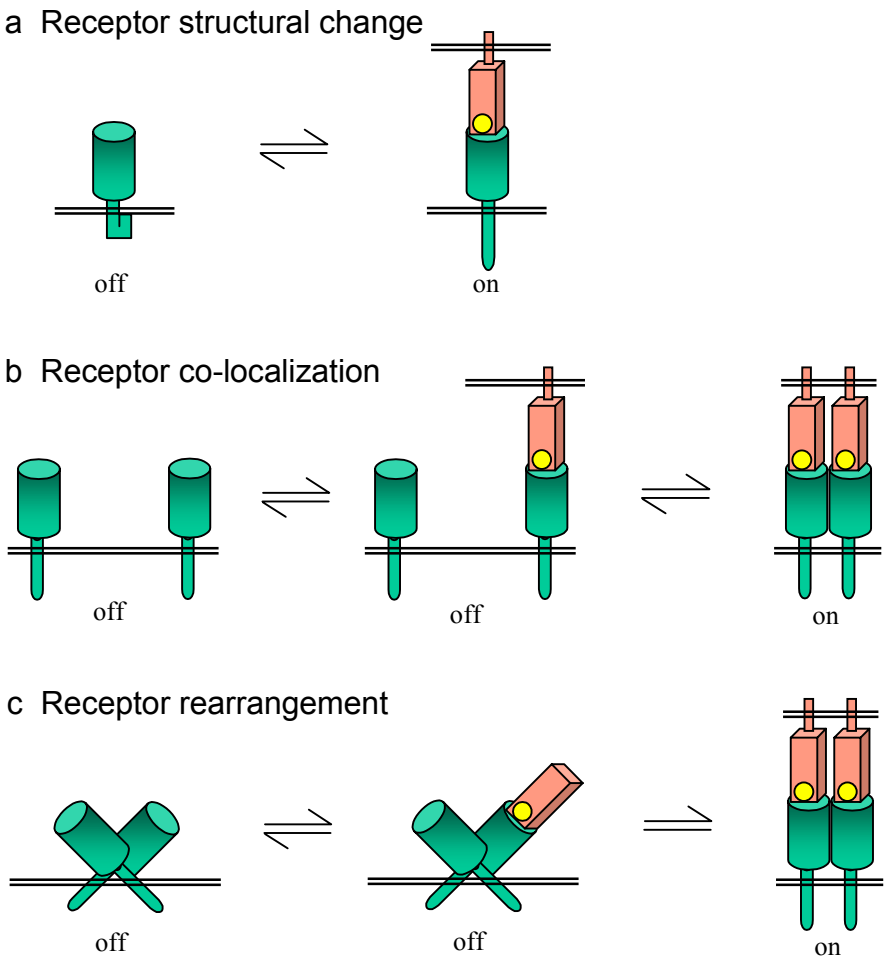


Figure V.1. *Mechanisms of transmembrane signaling.* (a) Ligand-induced conformational change, typified by seven transmembrane span receptors that activate G-proteins. (b) Ligand-induced receptor co-localization, typified by the fibroblast growth factor receptor tyrosine kinase, which is activated by dimerization. (c) Ligand-induced allosteric rearrangement, typified by the bacterial aspartic acid receptor, in which ligand binding induces a helical reorientation.

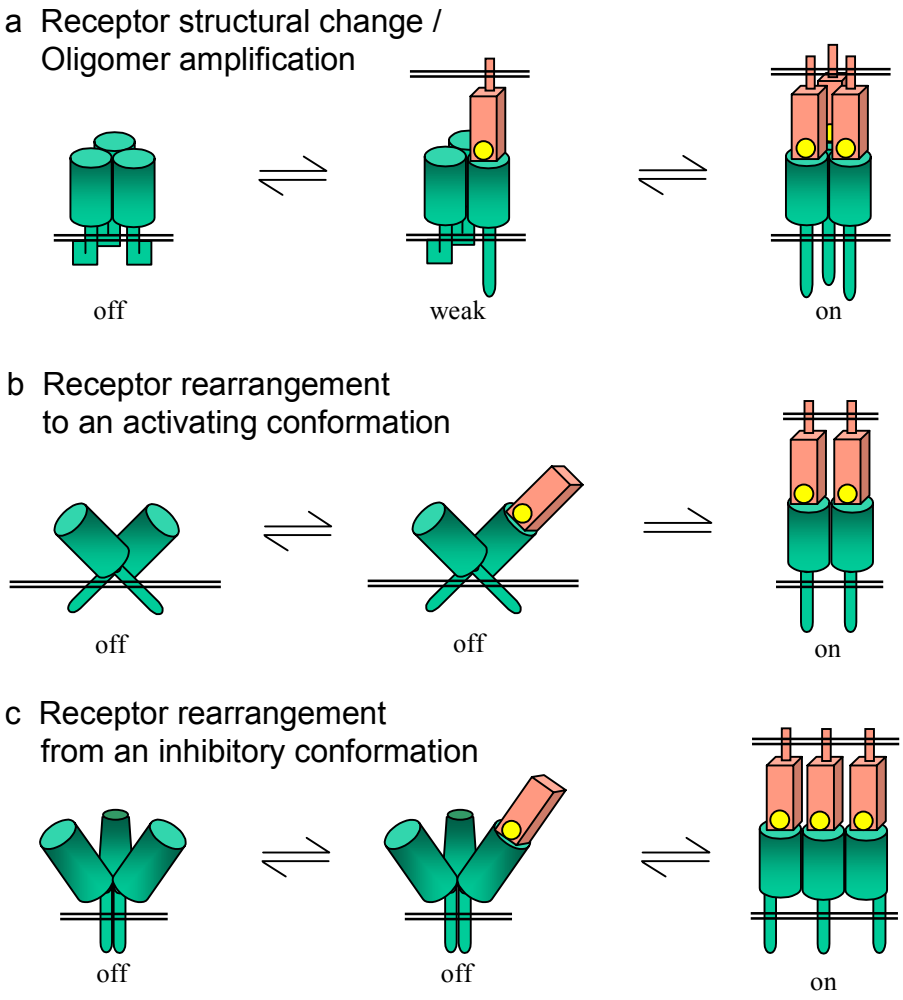


Figure V.2. *Alternative mechanisms of transmembrane signaling* (a) Conformational change / oligomer amplification model. Monomeric engangement of receptor triggers allosteric conformational changes in the cytoplasmic domain. Simultaneous engagement of multiple receptors amplifies intracellular signaling processes. Proposed by M. Reth, Trends Immunol, 2001 (b) Receptor rearrangement to an active conformation model Multivalent engagement of receptors causes reorientation of receptor subunits into particular activating conformation of intracellular domains. (c) Receptor rearrangement from an inbitory conformation model. Multivalent engagement of receptors causes reorientaton of subunits and disrupts a specific inhibitory conformation of intracellular domains.

VI. Conclusions and Future Directions for Research

VI.A. Summary of Thesis and Overview of Future Directions for Research

In chapters II and III I describe the production and use of soluble fluorescent oligomers of DR-peptide complex to detect antigen-specific T cells. This work establishes that DR oligomers can be used to identify many $CD4^+$ T cells, but that it should be kept in mind that some may escape detection. I concluded that detection depends not only on TCR-specificity, but also on cellular response, and that some T cells stain differently than others for reasons aside from the K_d of the TCR/MHC-peptide interaction. This suggests that DR oligomers can be powerful tools to study T cell avidity and the modulation of avidity between various T cell activation states. In Chapter IV I used DR oligomers along with IFN-γ surface capture assay to examine the TCR repertoire of Ha-specific DR1-restricted $CD4^+$ T cells. We found the response to be extremely diverse in both Vβ usage and CDR3 sequence. In Chapter V I described work from our laboratory investigating the triggering mechanism of TCR and discussed the implications of these results on various proposed models of transmembrane signaling.

The work of this thesis has stradled two major goals of current immunological research. One has been the goal of understanding the mechanism of lymphocyte activation, T cells in particular. The other has been a relatively practical and more medical goal of identifying and enumerating antigen-specific T cells in various disease states. Although future work inspired by this thesis will likely continue this dual purpose to some extent, many of the projects that I suggest below belong more to one category than to the other. I first describe ways in which MHC oligomers and similar reagents can

be used to further understand the mechanism of T cell transmembrane signaling and membrane-proximal activation events. I then briefly discuss further investigations of the one project that will likely continue to hold the interest of both basic-science and clinical immunologists, the analysis of the TCR repertoire specific for particular antigens. Finally, I switch gears to discuss potential technological improvements of current MHC tetramer technology.

VI.B. T cell activation projects

One of the central goals of our lab is to understand the mechanism of T cell activation. In addition to my own thesis work, I have been involved in the research of Jennifer Cochran, who used soluble DR-peptide oligomers of various sizes to probe the molecular requirements for T cell activation. Jennifer Stone and M. Todd Thompson are continuing similar research. Below I describe my thoughts on the future projects in this arena based on the work that we have accomplished so far and the current state of the field.

T cells can be broken down into many different subsets: naive and memory, $CD4^+$ and $CD8^+$, cytotoxic and helper, regulatory and non-regulatory. And even for particular kinds of T cells, many different kinds of response are capable, and, in fact, necessary: differentiation, anergization, tolerance, and various degrees of activation. During T cell development, the T cell receptor alternately serves the purpose of positive selection (productive TCR engagement leads to clonal survival) and negative selection (productive TCR engagement leads to clonal deletion). What factors regulate which T

cells respond in what manner at what time? This is a critical and yet unresolved question in immunology today.

VI.B.1 T cell avidity modulation

One potential factor for modulating T cell responses is TCR/MHC-peptide avidity. Because the TCR does not undergo somatic mutation, the affinity of a TCR on a mature T cell for a cognate ligand is fixed. However, because MHC-peptide exists multivalently by virtue of being membrane bound on an APC, the distribution and mobility of TCR on a T cell can greatly alter the number of TCR/MHC-peptide complexes engaged and the manner of engagement. This can be analyzed as the effective avidity of the TCR/MHC-peptide interaction. Work in our laboratory (Stone et al 2001) and from others (de Visser et al 2000; Fahmy et al 2001; Hesse et al 2001; Margulies 2001; Slifka & Whitton 2001) has pointed out the critical role of T cell avidity in modulating the response of a T cell. A variety of processes can alter the avidity of a T cell and, thus, modulate its activation character and/or threshold. As discussed in Chapter III, soluble MHC oligomers are ideally suited to the examination of T cell avidity modulation.

A study by Fahmy and coworkers (Fahmy et al 2001) has addressed T cell avidity modulation between naive and activated $CD8^+$ T cells in a TCR transgenic mouse, concluding that differential TCR oligomerization resulted in higher TCR avidity in memory T cells. I think that the work of Fahmy and coworkers should be replicated in both $CD8^+$ and $CD4^+$ naive and memory T cells. Some thought should be given to trying to conduct similar naive/memory studies in humans, although it may be difficult to obtain

controlled naive and memory T cell samples. Jennifer Stone and M.Todd Thompson in our laboratory are currently engaged in a collaboration with Drs. Jianzhu Chen and Herman Eisen in the Department of Biology to examine the activation mechanism of murine $CD8^+$ T cells using soluble class I MHC oligomers which address some of these concerns. However, because our previous work in human $CD4^+$ T cells is difficult to compare to murine $CD8^+$ T cells, we should begin projects to either investigate human $CD8^+$ T cells or murine $CD4^+$ T cells as well.

VI.B.1 Investigation of cytotoxic $CD4^+$ T cells

I think that possibly the most fruitful line of research to understand the difference between subsets of T cells may be to examine the difference between cytotoxic and helper $CD4^+$ T cells. Although classically $CD4^+$ T cells are defined as being helper T cells, there is a significant body of literature on the role of cytotoxic $CD4^+$ T cells in the immune response to disease (Norris et al 2001; Porakishvili et al 2001; Suni et al 2001). This provides a way to compare the cytotoxic and helper T cells without the complicating differences of CD4 or CD8 phenotype and class I or class II MHC restriction. Research from the past few years has suggested that nearly all $CD8^+$ T cells can be stained by MHC oligomers at either 37°C or 4°C (Whelan et al 1999), while my work has clearly shown that many $CD4^+$ T cells can only be stained at 37°C. Other studies have suggested that $CD8^+$ T cells and $CD4^+$ T cells might have significanlty different activation mechanisms; $CD8^+$ T cells appear to be activated by monomeric engagement of TCR (Delon et al 1998; Sykulev et al 1996), while $CD4^+$ T cells seem to require multivalent engagement (Boniface et al 1998; Cochran et al 2000). It is not clear whether cytotoxic

$CD4^+$ T cells will behave more like cytotoxic $CD8^+$ T cells or helper $CD4^+$ T cells with regard to MHC oligomer staining and TCR activation. If cytotoxic $CD4^+$ T cells appeared to behave similarly to cytotoxic $CD8^+$ T cells, it would be a very exciting result for immunology, and would make this system especially powerful for us because we would be able to study cells with identical cell-surface phenotype and ligand specificity, but different activation characteristics.

In order to begin this study, there are some simple pilot experiments to perform. I have previously isolated short-term polyclonal $CD4^+$ T cell lines specific for DR1-Ha, some of which are stored in our laboratory as frozen stocks and protocols for which are included in the appendices of this thesis. Preliminary reading in the literature has suggested that aside from CD4/CD8 phenotype, it is difficult to identify which cells of a mixture possess cytotoxic activity. Instead, their activity is typically detected by the death of target cells or the presence of certain secreted enzymes. Thus, my previously isolated Ha-specific, DR1-restricted lines should be tested in bulk for cytotoxic activity. If there is any cytotoxic activity within the polyclonal line, I think that the easiest further experiment would be to split the pool according to the ability to be stained by DR1-Ha oligomer at 4°C. This could be accomplished either by flow cytometry assisted cell sorting (FACS) or magnetic bead enrichment using anti-PE microbeads (Miltenyi Biotec). According to the hypothesis that cytotoxic $CD4^+$ T cells should behave like $CD8^+$ T cells, the cytotoxic activity should be entirely within the 4°C-staining population, and the 4°C-non-staining population will be capable of being stained at 37°C but will not contain any cytotoxic cells. Bulk cytotoxic assay of these pools should determine whether this is true or not.

If the above experiments yield interesting results, then many more experiments can be easily conceived on the 4°C-staining and 4°C-non-staining pools: detailed activation-threshold analysis using monomers, dimers, trimers and tetramers of DR-peptide, as in the work of Cochran, et al, 2000; determination of K_d and K_x values as in the work of Stone, et al, 2001; TCR repertoire analysis as in Chapter IV of this thesis. In addition, it would be powerful to expand the results to other clones, and to other epitopes. In particular, epitopes derived from HIV would be valuable, since cytotoxic $CD4^+$ T cells may play an important role in HIV pathology (Barouch & Letvin 2000; Norris et al 2001), and we have an established relationship with Philip Norris and Eric Rosenberg at Massachusetts General Hospital studying HIV-specific $CD4^+$ T cells.

Once we become convinced of the importance of T cell avidity modulation within the immune system, a very important question arises: What is the mechanism? I can envision three categories of mechanisms: differential localization of TCR in membrane domains (rafts), immobilization of TCR on the cytoskeleton, or changes in the static oligomerization state of TCR. However, these hypotheses can only be studied in detail once we have a controlled system to study and manipulate, which the above 4°C-staining and 4°C-non-staining pools might potentially be.

VI.B.2 Basis of T cell anergy

T cell anergy is defined as the state of a T cell in which it fails to proliferate in response to proper stimulation. It has been suggested that this state occurs from a biochemical block in one or several signaling pathways (Kimura et al 2000; Schwartz 1997; Sundstedt et al 1996; Utting et al 2000). Anergy is commonly induced *in vitro* by

providing a partial stimulus to the T cell, such triggering the TCR without proper co-stimulation. In Chapter II I briefly indicated that T cells anergized in this manner fail to be detected by DR-peptide oligomers. However, I have been unable to clearly assess the state of the T cells after such anergization protocols because of the complicating effects of TCR downregulation, apoptosis, and background proliferation caused by the anergization stimulus itself. In further research it has appeared that DR oligomer staining tracks with TCR surface expression levels, suggesting a very trivial mechanism for our observations.

Because T cell anergy is defined phenomenologically rather than biochemically, researchers inducing anergy by different methods may be studying significantly different phenomena. It may be that many of the anergic states detailed in the literature are caused by CD3 downregulation . It may also be that some of the researchers misinterpreted cell death as anergy in bulk cellular assays where they would not be distinguishable. Previous reports of T cell anergy utilize significantly different anergization protocols on various kinds of cells, resulting in divergent conclusions (Appel et al 2001; Greenwald et al 2001; Korb et al 1999; Powell et al 1999). I believe that it is likely that various biochemical phenomena have been historically referred to collectively as anergy. Furthermore, I think that the *in vitro* induction of anergy in human T cell clones and lines is a very poor model for the study of immunologically-relevant anergic states. Indeed, it has been observed that T cells stimulated by a variety of methods, including ones typically thought of to induce anergy, all similarly induce a period of T cell unresponsiveness (De Mattia et al 1999), suggesting that some of the previous studies of T cell anergy might have actually been describing a mechanism of immunological

homeostasis based, potentially, on TCR downregulation. If further work into the basis of anergy is to be perfomed within the Stern laboratory, I recommend that it be performed in the murine system where *in vivo* anergized T cells can be regularly obtained (Kimura et al 2000; Migita et al 1995; Mirshahidi et al 2001; Utting et al 2000) and we are more likely to learn something relevant to either TCR signaling pathways or immune regulation.

VI.C TCR repertoire analysis

VI.C.1 TCR Proteomics

In Chapter IV we used DR oligomers along with IFN-γ surface-capture to analyze the TCRβ sequences of $CD4^+$ T cell clones that responded to the Ha peptide bound to HLA-DR1. We argued that similar studies done on a larger scale using the tools of genomics and bioinformatics would define a new field of research, tentatively dubbed TCR proteomics. The goals of such a field would encompass both a basic understanding of the mechanisms that shape the TCR repertoire, as well as a detailed understanding of the T cell response to particular pathogens.

Several lines of experimentation should be followed in future research. The methodology used in Chapter IV should be expanded to more individuals and more sequences should be analyzed for each person. This will improve the statistics and strengthen the conclusions that have already been made. Further, the resting non-expanded repertoire of each individual should be examined in order to control for glitches in the methodology and for differences between the individuals unrelated to the peptide

being studied. Within this analysis, the other class II MHC alleles possessed by each individual should be evaluated for their potential contribution to the repertoires.

At the same time I would conduct some pilot studies examining other $CD4^+$ T cell epitopes. This will help us understand whether the diversity observed in our results is common to many class II MHC epitopes or unique to Ha. It might also be of interest to examine $CD8^+$ T cell epitopes in order to make some general observations about the $CD8^+$ and $CD4^+$ T cell responses. It would be especially powerful if all of these studies were carried out on epitopes derived from the same pathogen in order to control for the previous exposure of our volunteers. Influenza is a particularly attractive system to study because it is a disease of significant importance to human health and there is already a large amount of research mapping epitopes (Di Fabio et al 1994; Gelder et al 1998; Gelder et al 1996; Jameson et al 1998).

The payoffs of such research would be manifold. A thorough understanding of the breadth and variety of response to particular antigens by various individuals might aid the design of improved and streamlined vaccines, possibly incorporating such technologies as peptido-mimetic antigens. At the same time, such research would yield invaluable information to basic science about the formation of the TCR repertoire and the interactions of MHC with TCR. Finally, as in human genomics, I believe that a number of unexpected results regarding the TCR repertoire would be discovered by a comprehensive TCR proteomics research program.

VI.D. Improved MHC tetramer technology

In Chapter II we showed that DR oligomers are not simply probes for TCR specificity; that the response of the cell plays a critical role in the usefulness of this technology on $CD4^+$ T cells. Although previously unappreciated, reports have been surfacing suggesting that this phenomenon occurs with class I MHC tetramers on $CD8^+$ T cells as well (Bertoletti 2001; de Visser et al 2000; Moser et al 2001; Spencer & Braciale 2000). Even if exceptions to the rule are rare, by virtue of being unusual they may also be important. Phenomena which might cause altered MHC tetramer binding might include any phenomena known to obscure or blunt normal T cell functions, including T cell tolerance, exhaustion, suppression, and anergy, which have become increasingly important in immunological research. This points out the value in using MHC tetramers to identify unusual and potentially interesting cells.

However, the original goal of function-independent identification of antigen-specific T cells may not be best accomplished with current MHC tetramer technology. I see two potential options for further advances in the reagent.

VI.D.1 *Brighter oligomers*

One potential development is the use of brighter fluorescent labels to enable detection of previously undetectable cells. The only technology of which I am aware with sufficient potential to justify the effort of adaptation is that of semiconductor quantum dots (Chan & Nie 1998; Han et al 2001). Quantum dots (QDs) are colloidal semiconductor nanocrystals, ZnS-capped CdSe in one common formulation, and exhibit extremely bright luminescence with very narrow excitation and emission bands. Variation of size and surface conjugation can effectively tune them to a wide variety of

colors. Thus, the combination of QDs with MHC oligomers might enable the simultaneous analysis of many different peptides at once. However, the technology of solubilizing QDs in aqueous buffers and conjugating them to proteins appear to be in the early stages of development and so it may be some time before they are ready for use by immunologists.

VI.D.2 *Diffusable oligomers*

A second alternative modification of MHC tetramers is to conjugate them to lipid vesicles. One significant difference between MHC-peptide complexes on an APC and on a fluorescent streptavidin is that they diffuse freely in the two dimensions of the APC plasma membrane, but are fixed in the defined geometry of the streptavidin. It is possible that allowing the MHC-peptide complexes the mobility allowed in a membrane is critical for proper multivalent engagement. Fluorescent labeling of the vesicles could be achieved by loading them thousands of soluble fluorophores or by using fluorophore-conjugated lipids, potentially making them significantly brighter per MHC than the streptavidin-phycoerythrin based reagents used in this thesis. The primary stumbling blocks to the development of such technology would likely include devising a facile method to attach the MHC-peptide complexes to the vesicles, reagent instability, non-specific binding to surfaces and cells, and a lack of reactivity with T cells.

It is unclear to me that either of the above proposals are likely to be successful in developing MHC oligomer reagents that detect T cells better than the current streptavidin-based. However, the payoff of success may be worth the effort of investigation.

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Plasmid Prep: Large Scale Alkaline Lysis

Sep. 29, 96
-Tom

Day 1:
Inoculate 1 L of LB/Antibiotic.
grow overnight >16 hours at 37C. (up to 40 hrs should be fine)

Day 2:
Dispense into 500 ml centrifuge bottles, label bottles and balance.

try to keep each bottle at minimum volume because they sometimes lose some of the upper volume during centrifugation. Use the bottles with the rubber o-ring caps, if available. Label with very small pieces of tape and remove when done. Large pieces of tape will increase the bottle's radius and it will get stuck in the rotor. Balance to within 0.1 g. You can use DI Water to balance since you will be adding very little and the cells will still be approximately isotonic.

Spin 6000g 12 min. 4Celcius.

Decant supernatant into flask that you originally grew cells and Clorox it before you pour down the drain.

Resuspend pellet in 20 ml of Buffer P1 stored at 4C

(50 mM Tris, pH 8.0, 10 mM EDTA pH 8.0, 0.1 mg/ml Rnase A). Vortex very roughly. The cells are relatively happy in this buffer (although the lack of salt around may cause a few of them to burst but that's ok.) This buffer is mostly just to get them back into solution from the pellet. The Rnase isn't doing anything until the cells actually get lysed. You will find Buffer P1 in the fridge under the pH meter. Be sure you get a bottle that HAS had Rnase already added.

Add 20 ml of Room Temp Buffer P2. Incubate at RT about 5 min. (1 min is probably OK)

(0.2 M NaOH, 1% SDS) SDS is a detergent. OH and SDS will lyse the cells almost immediately. Shake gently in your hand to mix the solutions thoroughly. Do NOT vortex. Notice the stringiness of the solution. That is the DNA of the host E. Coli. If you vortex you will shear this DNA and it will copurify with the plasmid DNA. You will find some of this solution in my bay in a funny squat plastic bottle labelled with white tape.

Add 20 ml of Buffer P3 at 4C. Incubate at 4C for approx. 3 min.

(3.0 M Potassium acetate, pH 5.5, adjusted to pH with Acetic Acid) This neutralizes the pH of the solution so that the DNA doesn't hydrolyze and it precipitates the SDS. Shake solution with your hand to mix thoroughly but, again, don't shake or vortex violently.

Centrifuge as long and fast as is convenient.

At minimum, 30 min at 6000g will do OK if you want to use disposable 50 ml conicals. However, I suggest using 40 ml centrifuge tubes and the JA25.50 rotor and spinning at 75,000g for 20 min (be sure to balance within 0.05 g). You can use the same 500 ml centrifuge bottles at 10,000g for 30 min. if you're lazy. This step removes precipitate SDS, and cellular debris. I think most of the chromosomal DNA spins down here because it is associated with proteins and cellular debris.

Remove Supernatant carefully so as to avoid any solid material from the pellet.

I like to use a 25 ml pipet for this. Remove into 40 ml centrifuge tubes and be sure to leave enough remaining volume for the next step. If you have trouble getting rid of solid material, filter through cheesecloth (I have some in a drawer in my bay).

Add 0.7 volumes of Isopropanol (at Room Temp). Spin 30,000g for 20 min.

so if you have approx. 25 ml of solution (exact volume isn't critical but be within 10%) add 25*0.7=17.5 ml isopropanol. This precipitates all the nucleic acids (although you won't be able to see the ppt.). You can spin as gently as 6000g for 30 min. if you want to use 50 ml conicals but you're yield may be a bit lower, i'm not sure. Put the tubes in the rotor such that you can later remember which corner was the outside corner so that you know that is where the pellet will be. Mark with a marker if necessary.

Pour off the isopropanol

and use a pasteur pipet on an aspirator to remove most of the remaining isopropanol. Being careful not to touch the pellet! (this is why you were careful to remember which side of the tube was the outside!).

Rinse with ice-cold 70% Ethanol. Air dry for a few minutes. Dry in speedvac for 5 min.

There is some 70% EtOH in the -20C freezer door. Pour about 10 ml into your tube, carefully slosh it around and then dump it out and aspirate to remove most of the remainder. Dry in speedvac in the equipment room (ask tom how to use!). You will be left with a dry pellet (probably invisible) in the tube containing mostly plasmid DNA but also some small pieces of RNA and some contaminating chromosomal DNA and still some proteins.

Dissolve pellet in 400 ul TE (10 mM Tris pH between 7.2 and 8.0, 1 mM EDTA).

TE is the standard buffer for plasmid DNA. It keeps the pH slightly alkaline to prevent acid hydrolysis and the EDTA prevents stuff from growing in the solution. Transfer this solution to a 1.5 ml eppendorf tube.

Add equal volume (400 ul) of Phenol/Chloroform (50:50 mixture) (wear gloves!).

Vortex.

The phenol/chloroform will extract proteins from solution. The DNA will remain in the upper aqueous phase.

Spin in microcentrifuge (RT or 4C) 5 min max speed.

Using a 1 ml pipettor remove 90% of the upper, aqueous phase into a clean tube.

Be careful not to draw up any of the phen/chlor! (That's why we leave 10%!)

Add 0.1 volume (40 ul) 3 M Potassium Acetate pH 5.5 (I have some in a 50 ml conical).

Add 2.5 volumes (1 ml) of ice-cold 95% EtOH from the freezer. Cool in freezer 10 min.

This will precipitate the DNA to be sure we have avoided any residual Phen/Chlor. Whenever precipitating DNA with EtOH be sure to keep it as cold as possible all the time. The DNA needs to be heavily occupied by counter-ion in order to precipitate. that is why we added the 3 M Potassium Acetate.

Spin 10 minutes at max speed in the cold room.

place tubes in the centrifuge with the lid hinge pointing outward so that you know where the pellet will be.

Aspirate away the EtOH solution.

Add 0.5 ml 70% EtOH ice-cold. Vortex briefly. Centrifuge ice-cold for 8 min, max speed

This second wash with EtOH will remove much of the salt (Potass.Acetate) that we use to ppt the DNA)

Aspirate away the EtOH solution.

Dry in speed vac (while spinning) for 2 min. (until looks completely dry).

you can airdry this step (as you could the other time we used the speedvac) but I'm impatient.

Dissolve in 300 ul TE. Label your tube carefully for permanent storage.

Run 1 ul on an 1% Agarose gel to check yield and purity. Use lambda DNA markers.

Expect at least 1 ug per 1 ml of original culture. to you should have about 1 mg plasmid in this prep in 300 ul, approx 3 ug/ul. good!

Clean all your dishes! Especially be sure to clean centrifuge bottles very carefully. and caps too!

Rubidium Chloride Method for making Competant E. coli

-I got this procedure from Jeff Orr while he was in Williamson lab. It works very well for me.

1.Inoculate a single colony from a rich plate (Luria, Luria-Bertani) into 2 ml of rich broth (Luria-Bertani; RB) in a plating tube. Shake overnight at 37 C.
2.Subculture the overnight 1:100 in 1 Volume Unit of RB+20 mM MgSO4 (typically 250 ml). Grow to OD590=0.4-0.6 or Klett=60 (~2-3 h).
3.Centrifuge 5,000 rpm 5 min at 4 C. For 250 ml culture, use 2 250 ml centrifuge bottles, large rotor.
4.Gently resuspend pellent in 1/2.5 Volume Unit ice cold TFBI. For 250 ml subculture, use 100 ml TFBI; 50 ml/bottle. Combine the resuspended cells in one bottle. Keep all steps on ice and chill all pipets, tubes, flasks, etc. from this point on.
5.Incubate on ice for 5 min.
6.Centrifuge 5,000 rpm 5 min 4 C
7.resuspend pellet in 1/25 original volume cold TFB2. For 250 ml of original subculture, use 10 ml TFB2.
8.Incubate on ice 15-60 min. before aliquoting 100 ul/tube for storage at -70 C.
Quick-freeze the tubes. A convenient way to do this is to use ice-bath racks. These have a movable "lid" rack, with an ice compartment bottom (American Scientific Products, cat # S9233-1); set up the top-labeled tubes (open) in a rack with ice in the bottom compartment; distribute the cells; then close the tubes. In another bottom compartment, set up a dry ice/ethanol (or isopropanol) bath, wait for it to stop bubbling, then transfer the "lid" rack (which carries the tubes) to the dry ice bath bottom compartment for ~15 sec; drain the isopropanol, wipe with a tissue to get rid of the isopropanol, transfer to an empty bottom compartment and put the whole thing in the -70 freezer. After the tubes are well-frozen they can be dumped loose into a box or ice-cream carton, or transferred to slots in a storage box. Be careful not to get alcohol on the lips of the tubes. Liquid nitrogen can also be used, but not with these racks.
9.To transform, thaw an aliquot on ice; add DNA; incubate 1 h on ice; heat shock 45 seconds at 37 C; incubate on ice 2 min; dilute 15-fold into RB with no drug (for phenotypic expression); grow with vigorous aeration at 37 C for 20 min.; plate on selective medium.

This procedure works with most strains and should routinely give > 10E7 cfu/ug of pBR322 with reasonably healthy K-12 derivatives (using 0.1 ng/transformation). Frozen cells last at least a year.

Recipes

RB (Luria-Bertani medium)

per liter:
10 g Tryptone (Difco)

5 g Yeast Extract (Difco)
5 g NaCl
2 ml 1N NaOH

TFBI

30 mM KOAc (potassium acetate)
100 mM RbCl
10 mM CaCl2
50 mM MnCl2
15% glycerol

Adjust to pH 5.8 with acetic acid and filter (0.45 um, Nalgene units or Millipore filters) to
sterilize.

It is convenient to make this as:

5 g RbCl (Alfa)
12.3 ml KOAc 1 M
4.1 ml CaCl2 1 M
20.5 ml MnCl2 1 M (this is pink)
61.5 g glycerol
pH to 5.8 with ² 8 ml HOAc 0.1 M

make up to 410 ml; distribute in 100 ml sterile aliquots; and use 1 aliquot/250 ml culture.

TFBII

10 mM MOPS or PIPES
75 mM CaCl2
10 mM RbCl
15% glycerol

Adjust pH to 6.5 with KOH and filter to sterilize

Make up as

1.5 ml MOPS 1 M pH 6.5 (this is yellow)
11.25 ml CaCl2 1 M
1.5 ml RbCl 1 M
22.5 g glycerol
pH with 1 N K0H

make to 150 ml; filter; use 10 ml per original 250 ml culture.

Procedure adapted from one from the John Innes Institute (Norwich, England) via Joseph Utermohlen (Univ. of Arizona).

This procedure improves transformation with most strain backgrounds, when compared with CaCl2 procedure.

Note that Dam- strains (e.g GM2163) will yield 10-100-fold fewer transformants than isogenic Dam+ strains, probably due to poor initiation of the second round of replication. BL21(DE3) also transforms poorly with this and other transformation procedures.

Cloning:

Tips on cloning genes into plasmids

-tom, 2/7/97

Vector Piece:

Prep: Qiagen midi prep recommended (or something similar). Alk. Lys. is sometimes OK but I find some Alk Lys preps aren't digested easily. In this case, just QiaQuick 10 ug and you're set.

Digest: about 3 ug per attempted clone. Use "Restricition Site Frequencies in lambda DNA" in NEB catalog and calculate for about 3 fold xs enzyme units over DNA and digest for about two hours. These days I almost always use 1 ul CIP (Calf Intestine Alkaline Phosphatase, from NEB) in the digestion as well. Be sure total volume of enzyme added is less than 1/10th the total rxn volume, and keep DNA conc. around 0.2 ug/ul. If rxn volume is >30 ul, clean up and concentrate with a Qia quick to be able to load into a single well on gel. In my experience it is important to use enzymes that cut at least 75% in the RE buffer being used (see table in NEB). Otherwise, do sequential digests using a QiaQuick to change buffers. (Put CIP in at least the second digest, of course).

Gel Purify: Clean out gel box and gel tray. Pour a fresh 1% gel. Run 2-3 inches (four hours?) at 70 V. Look with the Stern handheld UV lamp or other very dim UV lamp in the darkroom and cut band carefully with clean razorblade into 1.5ml tube. Follow Qiaquick Gel Extraction directions. Elute DNA with 2mM (or 10 mM) Tris (pH 7.5-8.0) in 20-30 ul (be careful to carefully pipet the buffer onto the Qiaquick membrane, not the sidewall or lip). Run 2 ul of Gel Pure product on an agarose gel next to 5 ul of L5/HinfI digest to estimate concentration. I would expect about 0.5 ug total yield from 3 ug original uncut vector.

Insert:

From PCR Product: take 100 ul PCR product, estimate total ug by comparison with L5/HinfI on a gel, purify 3-10 ug on QiaQuick Column (Procedure when using "Gel Extraction" Kit: use 4-5 volumes QX1, spin thru column, wash with PE, spin dry in dry collection tube twice, elute with 50 ul water).

From another vector: cut 5-8 ug DNA (or less if insert piece is large). keep [dna] around 0.2 ug/ul.

Digest: Calculate cut sites per bp in your DNA and compare to Lambda DNA. Use approx. 4-fold xs Enzyme for 2-3 hrs. Note that this often requires large amounts of enzyme (and large volumes!) for PCR product. Use Qiaquick to concentrate the sample before loading on gel.

Gel Purify: run for 1-4 inches on an agarose gel. Depending on what you're doing, it probably doesn't matter so much whether it is 1% or 2%. Use Stern Lab handheld UV lamp in darkroom and cut out bands with a clean razor blade. Purify according to Qiaquick protocol (use 4-5 vol. QX1 for >2% gels, don't forget isopropanol wash.) Elute with 20 ul 2-10 mM Tris pH 7.4-8. Run 4 ul on agarose gel along with 5 ul L5/HinfI to estimate size and concentration.

Cloning from Primers: if you need to kinase them (ie if you used CIP in your vector prep), kinase them each separately and heat inactivate the kinase when done. mix equimolar at >= 10 uM. Buffer doesn't seem to matter much, but I would think 10 mM MgCl2, 50 mM NaCl, 10 mM Tris 7.8 is a good idea, or any RE buffer. Heat up to 90-100 celcius and allow to cool slowly to RT (you can use the PCR machine, or use a water bath or heat block and just turn it off when it reaches 90C or starts to boil. Once cool, store in freezer. When ready to use, dilute to 1-100 fM (ie 1-100 nmol/ul) and use 1 ul in ligation reaction.

Ligation Rxn:

Supposedly we're supposed to use 200 fmol of vector

2E-13 mol * 650 g/mol/bp * 6000 bp = 0.78 ug.

for a 6 kb vector.

Well, that seems ridiculous to me so I usually use about 200 ng for a new vector. I usually use about 50 ng for a tested/proved vector. And I usually use about 100 ng insert, or approximately 5-fold xs by moles (since often insert is about 1/10th the size of vector, 1/2 the weight is perfect).

Plan tubes of vector/no_insert, vector/various_inserts.

Set up ligation rxn in 12-20 ul with 0.5-1 ul T4 DNA Ligase (NEB, or Martha Rook). Leave at 16C for 4-24 hrs. (2-3 hrs is probably ok. For faster ligation RT is OK.)

Transform 2-4 ul into 100 ul DH5alpha, competant by Rubidium Chloride, or use commercially available competent cells (PGC Scientifics sells cheap DH5a comps).

Picking, Growing, Sequencing:

If you have *at least* 2-fold xs number of colonies over background (for, at least, most of your attempted inserts) then you can figure the cloning *may* have worked. Really good vector will give 10-20 fold xs (200 colonies with <10 background. Save that vector!).

Depending on how confident you are of the colonies, pick 2-5 colonies from each plate (include 2 from NoInsert) into LB/Amp culture.

Depending on how confident you are of the colonies, grow cultures of either 5 ml or 100 ml or 400 ml (more confident=larger culture).

Grow cultures overnight.

Freeze down 0.3 ml with 0.3 ml LB/30%Glycerol in -70%.

Do Alk Lys. prep or other plasmid prep. OverDigest approx. 2 ug (figure at least 1 ug per ml of culture) with the same enzymes you cloned with, run on 1% gel and look for properly sized insert. You may want to also do single-cut digest

and/or a digest that cuts in the middle of the insert. If you have lots of digests to do, use a Falcon rnd-bottom 96well plate.
If you have clones that look right, Qiagen purify 5-100 ug and send out for DNA sequencing!!!! Nice.

PCR screen for ligation colonies.

-Tom

easy screen! best for ligation results when signal:background is only 2 or 3.
Few false positives, but you can get plenty of false negatives.
Choose primers cleverly. You might want to even do two diff. primer sets.

Pick colony with sterile stick/toothpick.
- dip into 10 ul water in pcr tube
- dip into LB/amp culture
- streak on plate
- Name/number each culture/pcr to keep them all coordinated

Repeat for 5-20 colonies as you desire.
Put plate and liquid cultures to grow at 37C
Include one tube with 20 ng of +control template, and one with no DNA
Put PCR tubes to boil for about five minutes (or on PCR machine at 94 for 10 min)
Make Master Mix for PCR:
- Per tube (and do enough master mix for 1 extra)
  - 2.5 ul dNTP
  - 2.5 ul 10X buffer
  - 1 uM first primer
  - 1 uM second primer
  - H2O up to 15 ul per tube
  - .25 ul Taq or Pfu
- Aliquot 15 into PCR tubes
- Add 10 ul of 1:5 diluted boiled colony. If you want, you could try multiple dilution ratios.

PCR 30 cycles at something like 50C or 55C
- (ex: (30"94C,30"45C,60"72C)x5, (30"94C,30"55C,60"72C)x30)

Run 10 ul each on 2% Agarose gel. Be sure you at least get the +control to work if the others don't.

Note: that there are lots of games to play with the primers. for example, use one primer that is from insert and one from vector and that can give you the orientation of your ligation if you are doing a symmetric ligation! Or choose primers to give distinctly differently sized inserts based on what was cloned.

DNA Sequencing:

How Tom has learned to Sequence DNA: 5/17/95

My Version of the Sequenase Directions with a few comments.

Denaturation and Annealing: (about 2 hours)

Note: This takes the longest time of all. Use time to thaw and dilute reagents for later (but leave Sequenase in freezer). DNA is best if RNA free, but RNAase won't necessarily help. Boiling preps seem to work marginally. Wizard preps or PEG preps are best.

Trick Still to be Tried: if you have concentrated DNA (PEG prep or Boiling Prep), use 3 ul DNA, +.3 ul OH/EDTA, 30 min 37C, +.3 NaAc. Skip EtOH pptn! Just dilute up!

prepare primer at 1 uM (so 1 ul is 1 pmol)
take out the S35 now! (stored at -70C)
3-5 ug dsDNA +0.1 vol. OH/EDTA, 30 min., 37C (1-2 ug DNA should be OK).
+0.1 vol. 3 M NaAc (pH 4.5-5.5) + 3 vol. 100% EtOH, 15 min. -70C
spin max 10', wash with 70% EtOH, brief vortex, spin 6' aspirate
thoroughly, air dry 5'
take up in 7 ul water +2 ul Seq Rxn Buff +1 ul primer
heat at 37C for 30 min.
store on ice.

Reactions: (about 25 min.)

Note: Be prepared. Reactions must move QUICKLY.

Use the dGTP set of reagents.

S35-dATP must be stored at -70. It is good for two months after purchase. Note that the date on the bottle is 30 days after purchase!

For multiple samples best to use microtiter plate for the termination reactions. Use round bottomed 96-well plates. Use one of Mia's "sticky" plastic lids. No need to incubate at 37C if it is inconvenient. Use the bottom of a 85C heat block to denature the samples before loading the gel.

If using tubes for terminations, use a blue-tips rack. Fill up 37C water bath to appropriate height. Pre warm only 2-5 min. and spin down if time allows.

You can also freeze samples and run the gel the next day.

Keep reactions in GCAT order (like the MCAT).

Thaw termination mixtures (ddNTPs) on bench. Aliquot 2.5 ul into tubes.

Dilute Labeling Mix (dGTP, dCTP, dTTP) 1:5 with water.

Pre-mix 1 ul DTT, 2 ul Lab. Mix, .5 ul S35-ATP per sample (with some extra).

(+1 ul Mn Buff if you only need sequence up to 120).

Dilute Sequenase 1:8 with Gly. Enz. Buff. Save extra (stable!)

(Note: don't remove Sequenase stock from freezer.)

+3.5 ul of Labelling cocktail (DTT, dNTPs) into each sample.

Prewarm termination tubes.

+2 ul diluted Sequenase to each sample. 2-5 min. at RT

Stagger samples according to how long termination will take per sample. Allow 30 secs per sample if using tubes. Allow less for microtiter plates.

spin down termination tubes if you have time.

+3.5 of sample into each of its appropriate termination tubes.

Move quickly. Remember Hot Tips.

React at 37C 5-30 min. (Fine at RT).

+4 ul of Stop Solution. Timing no longer critical.

Freeze if desired (S35 good for one week. P32 should be used same day).

Heat to 75 (or greater) for 2 min. just before loading gel. Spin down samples. Load 3-5 ul.

On Assembling Gels: (about 20 min.)

The bigger plate has been Rainexed on the side opposite to the thermo-sticker. The smaller plate has also been Rainexed. Note you cannot load between two rainexed plates! Turn the small plate facing out.

Clean plates **VERY** carefully, finishing with a rinse of EtOH and long swipes with a chemwipe. Look carefully for remaining dust.

Methods: Clamps and ... Tape, Bottom Spacer, Bottom spacer of filter paper. Tape seems to not work well for sequencing gels. Bottom spacer is OK. Have it going all the way across the bottom so airbubbles don't get trapped. Use a dab of vaseline to help align the spacers.

Be sure to clamp on the spacers. Don't clamp beyond them.

Filter paper trick seems to work best. Use one long strips of filter paper (there may be some already cut in a drawer near the gel equipment.) Again, have it run the full length of the bottom. Don't remove! Electrophorese right thru the filter paper!

On Pouring Gels: (about 30 min., needs 30 min more to set)

Standard: 6% gel

15 ml 40% acrylamide mix (Appligene, buy in stockroom, store at 4C)

(20 ml for 8% gel. Better for longer sequencing?)

45 g Urea

5 ml 10X Gly. Tol. Buff.

water to 100 ml

filter, degas very briefly, using sterile filter unit. There should already be one labelled for sequencing with the gel equipment. Be sure to rinse out when done. Save 10 ml before you add APS and Temed.

+1 ml 10% APS, +25 ul Temed.

contain in a beaker convenient for use of 50 ml syringe.

Load gel using 50 ml syringe. Tilt gel up and slightly towards the corner where you are loading. Keep a constant stream. Adjust tilt angles to avoid air bubbles. (Rinse out syring when done and leave with Sequencing equipment).

Place horizontal (or nearly so). Insert backside of comb halfway into the oval cutout. For the small-well-combs insert less!

Use clamps over the combs! and push in the uppermost side-clamps.

Gel can be stored at least two days (probably a week) if top (and bottom if no bottom spacer or tape) is wrapped with a wet paper towel and saran wrap.

On Loading and Running Gels:

Trick: Use 1 M NaOAc in the lower reservoir chamber when you load your last set of samples (or before you prerun if you're only running one). This will compress the lower bands and give you the effect of having wedge spacers.

Load 1X Gly. Tol. Buff. into bottom reservoir.

You will need 1 L of Gly. Tol. Buff. for whole setup.

Load gel. Clamp it down.

If you had a bottom spacer, insert gel into buffer slowly and at an angle. Watch for catching bubbles. Then clamp the gel. Use a syringe with a bent needle (low gauge) to push the bubbles to one side or out).

Clamp down the drain for the top reservoir!

Be sure to put foam squares above the small plate on either side of the top reservoir!

Load 1X Gly. Tol. Buff. into top reservoir until above the small plate.

Blow out wells with syringe (without comb in there!).

Prerun at 65 W for 15+ min. (without comb)

Blow out wells again.

Arrange comb so it is barely poking into gel.

Load some dye in alternate lanes. Look for leaky lanes.

Use normal pipet tips and push up against the larger plate when ejecting. The sample will fall into the well on its own.

Run for about 2 min. in order to make space for your sample.

Heat your sample to 72C (or a little higher, up to 85C?) for 2 min. Put on ice. Spin Down. Load 3 ul in large well, 1 ul in small. GCAT (like MCAT).

Run at 65W (50-55C). Should take about 2.5 hours for a 6%?

You may want to load samples a second time after about 1.25 hrs. if you want to get a lot of sequence (and you have room).

You're done when lower dye (of the last set of samples loaded) runs off.

On Drying and Exposing Gels:

Note: Williamson lab does not like acetic acid on their gel dryers (ie fixed gels).
Cut a peice of gel-drying filter paper to fit the gel (with 1" extra on all sides).
Drain top reservoir of buffer. Remove plates onto bench paper.
Pull out spacers. Use a razor blade to pry apart plates.
Quickly press filter paper over gel and lift the far edge towards you. The gel will stick and look great (Lihui's trick!).
Quickly cover with saran wrap (This way the saran wrap will come off more easily once dried). Trim excess saran wrap (do NOT fold under).
Dry on Williamson dryer. Be sure to write down in log book. Be sure to turn on the filter (on the floor to your left). Use 80C, full vacuum for 20-30 minutes.
When dry, try to pull off saran wrap. (Don't force too much.)
Assuming you did manage to pull of saran wrap, spread around a little Talc (baby powder, with gloved fingers) so that gel doesn't stick to film.
Put gel inside autorad cassette with film. Expose, RT (1 day w/o saran, 2 w/).
In Dark room, under red light, remove film and put in wire holder. Put in developer (left reservoir) 1 min., check for bands under red light, Rinse in central reservoir thoroughly, put in fixer (right reservoir) 3 min., rinse in central reservoir thoroughly. Turn on lights and hang to dry.
Read sequence or start over!

Buffers:

10X TBE (1 L):	20X Glycerol Tolerant Buffer:
108 g Tris Base	216 g Tris Base
55 g Boric acid	72 g Taurine
20 mM EDTA	10 mM EDTA

Fermentation of E.coli in the Stern Lab:

Short Procedure for using the old New Brunswick Scientific Fermentors in the Stern Lab so that you can start at 3 pm on Day 1 and be done by 3 pm on Day 2.
-Tom

Day 1:

Make sure fermentor is clean.
Put 10 L worth of dry LB into bottom of Ferm Vessel
(I usually weigh into a 1 L beaker)
Fill with about 10 L of H2O (tap water is fine)
(use the marks on the side of vessel with the top assembly in)
Piece together assembly. Cover Openings with foil.
Leave the cap of the fat opening loose and leave the clamp on the hose drain open.
Prepare an 500 ml or 1 L Erlenmeyer flask with 200 ml LB for the o/n culture.
Wrap a large funnel with Alum Foil.
Make 20% D-Glucose (Dextrose) (500 ml)
It won't fully dissolve until autoclaved.
Autoclave Vessel, small culture, funnel, and Glucose 30 min Liquid Cycle.
Leave Caps Loose!
be sure Glucose and fermentor are in a 2ndary container with some water to reduce risk of cracking.
Prepare >=10 ml Ampicillin, 50 mg/ml, syringe filter sterilize, freeze.
Prepare >=10 ml IPTG, 0.5 M, syringe filter sterilize, freeze.
Be sure we've got some Antifoam. (Sigma, usually kept near ferm, but ask around).
Be sure we've got some sterile tips and pipets.
Cool the Small Culture on bench and then on ice.
Place the Vessel on the fermentor.
Insert Air-In probe, connect Air-Out venting,
When small culture is cool (<37C), add Amp to 50 ug/ml, Glucose to 0.2%, Inoculate from frozen E. coli stock, and shake at 37C o/n.

Day 2:

Arrive early. Start the Fermentor spinning.
Remove some of the LB and check pH to be certain that it is close to 7. Adjust if necessary.
Add about 400 ul Antifoam, 100 ml Glucose, 10 ml Amp to vessel
Tighten the hose-drain and the fat opening.
Fill the thermocouple well with water and put in the thermocouple as well as a second thermometer for you to monitor the temp yourself.
Turn on water and air (behind ferm). Start air blowing and water circulating.
Trick for Hot Water line: The water should be flowing quickly in the recirculation lines. Before you attach the lines, fill tubing in vessel assembly with water and squirt water into the lines. attach lines. With power off, turn on water behind ferm. Then turn on power and heat. Hopefully, you should see bubbles blow quickly out of the lines and it will sound relatively quiet. If you hear a loud intermittent motor noise, it probably isn't working. turn off heat and power before turning off water.
Turn on power, heat to about 35C, drive to about 500, cooling water
Adjust air flow to about 15 and 15
Check warm water lines to be sure they are heating up properly.
Check OD of o/n culture. Should be > 1.5, for certain, probably around 3 or 4.

When Fermentor is at a 33-37C, inoc. with 200 ml o/n culture.
In 2 hours check OD
Trick: OD600 of LB is about 0.07 when zeroed on air.
Between 2-3 hrs the OD600 will reach 1.2-1.5.
Remove 1 ml for a non-induced sample. Spin 30 sec max in eppendorf, aspirate off sup, resuspend in 100 ul reducing Urea loading buffer, vortex and boil.
Add 10 ml IPTG.
Add 200 ul Antifoam if necessary.
Check pH of culture (Optional).
Remove 10 ml into conical tube (use sterile pipet) and check on pH meter.
Carefully pH the 10 ml to 7.0+/-0.1 with base (KOH or NaOH is OK). (It should take ballpark 10 ul of 1 M base).
Scale up the base addn to the fermentor using 10 M base. Afterwards, check 10 ml again and try to keep pH between 6.8 an 7.2, or, at least, closer than it was before.
Let Cells Grow for 2-3 hours.
Periodically check foaming. Add more Antifoam if necessary.
Periodically check pH (optional).
Periodically check OD (dilute so that OD is <1.5 to get a proper reading).
I have found that by adjusting pH the cells grow to a max of about 5.5-6 ODs.

Alternate pH-control approach: the Williamson lab had a pH probe that was set up to regulate a peristaltic pump for automated addition of base during a fermentor. This works well but may take some work to set up. They had a long needle the poked through a septum and into the solution for base addition.

Collect cells:
Trick #1: Syphon. attach a long piece off tubing to the end of the hose drain so that it reaches about 10 inches from the ground. Open the valve and start the syphon by temporarily pinching the Air-Out and tightening the fat opening cap. Drain into 5 L Grad Cyl or into 4 L plastic bottles (old EtOH bottles).
Trick #2: Blow it out. Open hose-drain valve and force out culture by tightening the fat opening and pinching the Air-Out line. collect into 5L Grad cyl or 4 L plastic bottles (old EtOH bottles).
To get the last 500 ml, remove vessel from fermentor and take out the top-assembly and pour into container (use funnel for the narrow-mouthed 4 L bottles).
Spin down at 4-5000xg 10-15 min. Be sure to use 500 ml Centrifuge bottles with O-rings and use caps on the rotor buckets ALSO OR use 1L bottles and swinging bucket Sorval centrifuge in Ram's lab.
Collect repetitively into same six bottles. Chlorox the supernatants.

You're done!

Proceed with Inclusion Body Prep or other procedure as necessary.

Inclusion Bodies Prep:

Isolation, Washing, and solubilization of Inclusion Bodies from E. coli.
From Mia Rushe, based originally on preps from Don Wiley's lab.
-Tom

Solutions:

DNAse/RNase Solution

75 mM NaCl
50% glycerol
2 mg/ml DNAse (Sigma D-5025), 0.5 mg/ml RNase

Sucrose Solution

50 mM tris pH 8.0
25% sucrose
1 mM EDTA
.1% NaN3
10 mM DTT **(add fresh just before use)**

Deoxycholate/Triton

1 % Na Deoxycholate
1 % Triton x100
20 mM tris pH 7.5
100 mM NaCl
.1% NaN3
10 mM DTT **(add fresh just before use)**

Triton Solution

.5% Triton x100
50 mM tris pH 8.0
100 mM NaCl
1 mM EDTA
.1% NaN3
1 mM DTT **(add fresh just before use)**

Tris Solution

50 mM tris pH 8.0
1 mM EDTA
.1% NaN3
1 mM DTT **(add fresh just before use)**

Urea Solution

8 M urea
20 mM tris, pH 8.0
10 mM DTT **(add fresh just before use)**
.5 mM EDTA

4 M MgCl2
.5 M EDTA

*Protocol for pellet of 10L. cultures
In general, all steps are done best dilute.

1. Resuspend all pellets with total 200 ml sucrose solution, using a little extra to rinse all containers and pipettes. Collect all cells into a single or into two cent. bottles. with stir bar. Use a rubber policeman if you pelleted in a fixed angle rotor and then homogenize with chopper. If you have a flat pellet from a swinging bucket rotor, I usually have good luck shaking the bottle vigorously by hand to resuspend the bugs, no homogenization necessary.
2. Add approx 1 mg dry lysozyme per ml bug suspension and stir for 10 min (not necessary for BL21 pLys strains). Add 500 ml deoxycholate/triton solution (2.5 ml/ml suspended bacteria). Solution will get very viscous due to cell lysis and the release of DNA.
3. Add 4 M MgCl2 to make 5 mM final and 1 ml **DNAse/RNase solution** and stir 10 min. Wait until the solution becomes the viscosity of water. Give it a few minutes extra, then freeze at -20^{o} C overnight or until ready to complete prep.
4. Thaw solution thoroughly in 37-45 degrees celcius. Swirl to keep temperature distributed. Add 2 ml .5 M EDTA and stir. Spin at 8000 RPM 20 minutes (be sure to use bottles with o-ring tops!).
5. Carefully pour off supernatants. Expect pellets to be white and spread all along the outer wall of the bottle. Resuspend pellets in 100 ml triton solution. Use rubber spatula to get all of the pellet into the solution. "Chop" well. (20-30 seconds on nearly max speed of a Polytron homogenizer). Keep everything on ice as much as possible for all the washes in steps 5 and 6. Spin at 8000 rpm for 10-20 minutes. Repeat this 3 **more** times.
6. Repeat this wash three times with the Tris solution. You want to wash away ALL of the triton before resuspending in urea.
7. Dissolve the pellet in 30-50 ml urea solution. I have sometimes chopped in order to resuspend in urea, sometimes just gently shake 20 min. Dounce homogenizer is probably best. Transfer to 40 ml centrifuge tube or 50 ml corning tube.
8. Spin at 6k-15kxg 30 min. at 12-24^{o} C. Transfer supernatant to 15 ml or 50 ml conical tubes. Save 100 ul in an eppendorf tube for UV-Vis and SDS-Page analysis. Freeze at -70^{o}C in a well labeled box until ready for use. Limit the amount of time protein is in urea and not frozen. Make small enough aliquots so that you don't need to freeze/thaw later on.

Urea HQ Purification of Inclusion Bodies.

Tom Cameron, Stern Lab, MIT, modified from procedure of Mia Frayser (Mia Rushe), Stern Lab.

First: Make "crude" inclusion bodies and run them on a gel to make sure they are what you think they are!

Solutions:

1 M DTT in water. freshly dissolved or freshly thawed.

Lots of Urea! The volume of urea that you will need depends on the size of your column and number of runs, but make a lot! I often make between 5 L of 8 M urea. We use relatively crude, cheap urea from JTBaker, 4204-09, and while it dissolves we simultaneously deionize using beads from Sigma, M8157 "Mixed Bed Resin TMD-8". Urea is difficult to get into solution because 8 M is near saturated at RT and the solubilization of urea is endothermic. I find that the most convenient way to make Urea solutions is to weigh out urea, add water to approximately the final volume in erlenmeyer flasks, and shake on a shaker table at 37C only until it dissolves. Alternatively, sometimes I use a 5 L bucket, add about 2.5 L water, get it stirring, slowly add the correct amount of dry urea (it is hard to get the solution stirring without doing it this way). In order to keep the solution near RT I usually place the 5L bucket in a secondary container of warm water while stirring. Either way, we end up with 8 M urea containing deionizing beads. Remove the deionizing beads by filtering through filter paper in a buchner funnel. If you use Ultrapure urea, you can skip the deionizing beads. Also note that Urea is not very stable, especially at elevated temperatures, so once it dissolves, use it!

After filtering away the DI beads, then add other stuff to make …

Buffer A: 8 M deionized urea, 20 mM Tris pH 8.0 or 9.0, 1 mM DTT.

Buffer B: 8 M deionized urea, 20 mM Tris pH 8.0 or 9.0, 1 M NaCl, 1 mM DTT.

For DR-alpha, use pH 8.0. For DR-beta, use pH 9.0. DR-beta won't stick to the ion-exchange resin at pH 8.0, but it is generally best to keep pH as low as possible to prevent side-reactions occuring on the free cysteines of the subunits.

Urea solutions are unstable. They should be made fresh every day or else frozen. If you want to save time, it is probably okay to store them overnight at 4C, but I don't recommend it.

Filter Buffer A and Buffer B through 0.2 microns before using on the HPLC. I have been known to skip this step when I was lazy to save time, but it is probably not a good idea.

Pack a high-pressure column with HQ resin: Poros 20 HQ, available from Boehringer Mannheim. Assume that the column binds approx 2 mg protein per ml of bed resin. Column, once packed, can be reused for many years. Regenerate between uses by washing with 1 M NaCl/1 M NaOH (wear goggles!).

Procedure:

Before loading your subunits, reduce your subunits with 50 mM DTT for 20 min, RT.

Load 2-5 mg protein per ml of bed resin. You may want to dilute the subunits with buffer A to make sure that the conductivity is low as you load. Load relatively slowly. Elute with a shallow gradient (0 to 0.5 M NaCl over 10 or 15 column volumes, and then quickly up to 1 M NaCl). Collect lots of fractions. Repeat loading and elution with identical procedure several times so that all the fractions are equivalent. Pool fractions into 3-5 different pools. Save 100 ul from each pool for UV/Vis and SDS-Page and test-folding analysis. Aliquot pools in 15 ml or 50 ml tubes and freeze immediately. Take UV scans of various pools and look at the ratio of OD260:OD280. In general, I assume that the earlier fractions will fold better, but that isn't always true. You will probably have to test yourself!

Important:
Do not let the protein sit in thawed urea for long. Once purified, freeze. Once thawed, use. Absolutely do not ever let protein sit overnight in urea. Don't even let it sit for a few hours. And when you thaw it, don't let it get too warm!

Folding and Purifying DR1 with Peptide

Tom Cameron, Nov 2000

This procedure is a slight variant of the one developed by Mia Rushe (Frayser) and published in Protein Expr Purif. 1999 Feb;15(1):105-14.

Things you will need:

Reduced and Oxidized Glutathione (GSH and GSSG, from Sigma)
Glycerol (VWR Stockroom in Bldg 18).
DR subunits and Peptide
Dialysis tubing, wide. I use Spectra/Por-1, MWCO 6-8,000, flat width 40 mM (from VWR), soaked in water/azide overnight. You will need about 22 inches per "4L 5X" folding.
Appropriate vessels, stirbars, buchners, Whatman filter paper.
Filter Flasks of various sizes.
5 M NaCl, 1 M Tris pH 8.0, 1 M DTT, 0.5 M EDTA.
Filters, 0.2 or 0.4 um, and a clean reusable filter unit.
Lots of beakers and erlenmeyers.
Time of the HPLC (about 2-4 hours).
A column of Poros HQ-20 resin for use on HPLC (1.5 ml size is adequate, 4.6 mm x 7.6 cm)

Setup the Folding Reaction:

Day 1:

Decide on scale: Expect a final yield of about 1% for good peptides. If I want >= 1 mg of protein, I typically setup a "4L 5X" folding, which is 10 mg/L each subunit (total 40 mg each subunit, 80 mg total DR, 2 uM). Our standard protocol defines "1X" as being 2 mg/L each subunit (400 nM).

Use Good Subunits: The most common cause of a bad folding is starting with bad DRα or DRβ Be sure that after you HQ purified your subunits, test them in small scale folding reactions and assay them by ELISA. Throw out the bad fractions! I usually perform test foldings by putting 5 ml folding mix in a 15 ml polypropylene tube, add a good peptide (HA, to 200 nM) and while vortexing the tube, quickly shoot in subunits in urea. Then store 4C 2 days and assay 50 ul, 10 ul, 2 ul, 0.5 ul on an ELISA with appropriate standards.

Ahead of time, in a 4 L opaque plastic jug (either narrow mouth or wide mouth), prepare 25% glycerol mixed with MilliQ deionized water (w/v) and cool in cold room.

Add 0.5 mM EDTA, 10 mM Tris pH 8.5, 1 mM GSH (reduced glutathione), 0.1 mM GSSG (oxidized glutathione), and peptide.

Amount of peptide to use: For medium-strong binding peptides (Kd < 200 nM) try to add peptide to 200 nM or higher. For weak binding peptides, add up to 2 uM if you can spare it.

Thaw DRα and DRβ.

Set solutions stirring at a high speed while cold (either in cold room or in a ice bucket with ice).

Drip subunits into solution. Should take several minutes.

Techniques: you can setup a rubber stopper that fits the vessel with a needle, a stopcock and the barrel of a 30 ml or 60 ml syringe and use this to slowly drip subunits, or you can just manually drip the solutions from either serological pipets, pasteur pipets, or syringes with needles.

Continue stirring solutions for >= 5 minutes.

Store cold for >= 48 hours.

Purify:

Day 3:

Remove stirbar from solution. Absolutely do NOT stir the solution after you've added the DEAE beads, because the beads will get crushed and will be very difficult to filter.

Cut Dialysis tubing and put in water with 0.1% NaAzide to soak overnight.

For a "5X" add 2.5 g of dry DEAE Sephadex A-50 per liter. For a "1X" add 1 g/L.

Wait a few hours to let beads swell.

Gently invert bottle several times to resuspend beads and allow them to "see" the entire solution.

Optional: Repeat inversion several hours later, or next day.

Day 4:

Optional: Let beads settle overnight.

Collect beads by passing solution through a buchner funnel with Whatman filter paper. Take care that the filter paper has no gaps so that none of the beads flow through. For a "4L 5X" I find a 9 cm filter paper with appropriate sized buchner to be perfect. Be sure that you have adequate vacuum for the job. It will take a very long time if your vacuum is too weak.

Optional: If the beads are settled when you're ready to collect them, you can either siphon or aspirate away the upper buffer in order to save time collecting the beads.

If you're purifying multiple peptide foldings at once, you can set up a "manifold" of buchners and filter flasks and do all at the same time.

Wash beads with about 200 ml 20 mM Tris, pH 8.0, 1 mM DTT. (Can be skipped if you're in a rush).

Elute by adding NaCl for a final concentration of 0.6 M. I find that 10 g of DEAE beads hold about 160 ml of buffer, so add about 20 ml of 5 M NaCl and immediately stir with the bulb-end of a transfer pipet until solution is well mixed and watery (sp?). Let sit for >= 5 min.

Collect eluate using vacuum.

Filter eluate through filter paper again to be certain all beads are gone. (If any beads remain, when you switch the buffer into low salt they will bind up DR again!)

Prepare large vessel (14 L glass "beaker") with 20 mM Tris pH 8.0, 1 mM DTT for dialysis.

Day 4/5?

Set eluates to dialyze. Stir as vigorously as possible.

Dialyze >= 2 hrs (cold if > 4 hours). *If you are working with weak binding peptides, I suggest trying to purify via HQ same day you collect the DEAE beads.*

There is probably cloudy ppt in the dialysis bag. Spin solution in oakridge centrifuge tubes 20 min at 12,000xg.

Filter through 0.22 or 0.4 micron filters. Add extra DTT (to 4 mM) if you are using DR with a c-terminal cysteine (ACL, ACS, BCL or BCS).

Prepare Perseptives HPLC with your Poros HQ-20 column (about a 1 or 2 ml bed column will be plenty large). Use filter and degassed buffers, A: 20 mM Tris pH 8.0, B: 20 mM Tris pH 8.0, 0.5 M NaCl.

Clean column briefly with 1 M NaOH/1 M NaCl (load through line F) and wash subsequently with Buffer A (first through line F, then through line A).

Load sample through Line F. Mix with buffer A to keep the conductivity < 70 mM.

Run a program with a wash in A for 10 CV (reset frac. Collector, zero UV detector), then a gradient from 0 to 50% B in 20 CV, 50 to 100% B in 3 CV, hold at 100% B 3 CV, wash back into A for 10 CV. Collect fractions from the beginning of the gradient to the end of the 100% B step.

Expect DR-pep to elute at approx 120-200 mM conductivity. There will probably be another peak that elutes at high conductivity (I think this is peptide, but I'm not sure) and there will be a small peak at very low conductivity. Pool appropriate fractions. If appropriate, add cysteine-reactive reagents at this stage (ex: biotin-maleimide) and react RT 30 min. Dialyze to remove excess.

Concentrate protein in Centriprep-10 or Centriprep-30. Spin filter and use OD280 to quantitate protein (1.25 OD is 1 mg/ml for DR1).

DR ELISA

Notes by Tom, July 1999 based on Mia Rushe's protocol.

Solutions and Reagents:

PBST or TBST: PBS or TBS with 0.05% Triton X-100.
Blocking Solution: 3% BSA in PBSZ (0.1% Sodium Azide)
Dilution Solution with Z (DlnSlnZ): Mix 5 ml Blocking Solution with 45 ml PBST.
10X ABTS buffer and ABTS tablets (from Boehringer Mannheim).
Various antibodies: LB3.1, CHAMP, goat anti-rabbit HRP.
Immulon-4 HBX Plates, from Dynex, Cat # 3855, Phone 1 800 336 4543

Procedure:

-Coat plate with LB3.1 or L243, 100 ul of 2 ug/ml per well (200 ng per well). Incubate 37C 2 hours or 4C overnight.

-Wash each well three times with PBST

-Fill wells to top (about 375 ul) with Blocking Solution and incubate > 1 hr. At this stage, plates can be taped up, wrapped with saran wrap, and stored at 4C indefinitely.

-Prepare standards of DR, 1:2 dilutions from 100 ng down in DlnSlnZ. Prepare dilutions and duplicates of samples in DlnSlnZ.

-Dump Blocking Solution from plate, blot dry on paper towels, and add 100 ul of sample/standard to appropriate wells. Be sure that you have at least two wells with no DR. Incubate 37C 1 hr or 4C overnight

-Wash plate three times CAREFULLY.

-Prepare 10 ml of 1:50,000 CHAMP rabbit anti-DR sera in DlnSlnZ. Add 100 ul to each well. Incubate at least 30 min 37C.

-Wash 3 times.

-Prepare 10 ml 1:4,000 goat anti-rabbit HRP (peroxidase) conjugate in PBST (be sure there is no azide in this step!) and add 100 ul to each well, incubate 37C >= 20 min.

-Meanwhile, thaw five ml of 10X ABTS buffer, dilute with water and dissolve one ABTS tablet.

-Wash plate three times.

-Add 150 ul or 200 ul ABTS sln to each well. As it developes, start the ELISA reader and read results at 405 nm.

Biotinylation assay for DR1:

Sep, 2001, Tom Cameron.
Very nice assay, although it uses quite a bit of protein. Can probably be easily adapted for other proteins.

Materials:

Pour a 12.5% SDS-Page gel.
Streptavidin (>0.5 mg/ml). (best reagent is from Prozyme Inc, Cat # SA-010).
Get 5X SDS Reducing Loading Buffer (SDS R-LB), keep cold.
Ice bucket with ice.
PCR machine or Const. Temp. Block or Boiling water bath.

Procedure:

Aliquot 9 ug of DR1-biotin into a tube. Add 10 mM Tris pH 8 to final 30 ul.
Chill DR sample.
Add 7.5 ul cold 5X SDS R-LB. Pipet mix.
Aliquot 12 ul of this DR solution into three different pre-chilled tubes.
Boil tubes #2 and #3 2 min (>90C in a PCR block is fine). Keep #1 on ice.
Chill tubes #2 and #3 2 min.
Prepare some a solution of cold SA in SDS R-LB (don't boil).
Add 2 ug of SA in SDS R-LB. Pipet mix. Keep cold.
Load gel: MW markers, 2 ug free SA (NB), #1 (NB DR), #2 (B DR), #3 (B DR + SA).
Run gel 120 V for approximately 80 min.

Staining CD4+ T cells with DR Tetramers: Detailed step-by-step

-tom cameron, Dec 2000

Reagents:

DR1pep: Biotinylated DR1-peptide of choice in PBS.
SAPE: R-phycoerythrin conjugated Streptavidin from BioSource, Inc
RPMI and T cell Media
T cells and/or PBMCs
CD4-APC, CD14-PerCP (optional, good for PBMCs), CD3-Fitc (optional, but good, esp for clones).

Make the Oligomer Reagent:

For 50 ul:

Aliquot 1.5 ug DR1pep into a epp. tube
Dilute 2 ul SAPE with 4 ul PBS
Add 2 ul of diluted SAPE to DR1
Wait 2 min
Add 2 ul of diluted SAPE to DR1
Wait 2 min
Add remainder of diluted SAPE to DR1.
Wait 2 min.
Dilute DR1-SAPE oligomer with RPMI or T cell media to a final volume of 50 ul.

Notes: I call this a 60 ug/ml reagent, half of it is (by weight) DR, and approximately half is SAPE. Since SAPE is a poorly characterized heterogeneous reagent, that isn't very precise. I have determined experimentally that a final ratio of 1.3 ul of SAPE per 1 ug of DR1-pep gives optimal staining when using SAPE Lot 1401 from BioSource. Different lots of SAPE should be titrated with DR1pep to determine optimal ratio (or contact me and ask if I've done that experiment!) although approx 1 ul SAPE per 1 ug DR generally seems pretty good.

Stain the Cells:

Collect cells in a 15 ml conical tube and spin down 5 min 1500 rpm
Aspirate as much of the media as you can.
Resuspend the cells in a very small volume of media (10 ul for every stain that you want to do. So if you're doing only one specific tetramer, and one control tetramer, then resuspend in 20 ul).
Aliquot 10 ul into wells of a rnd bottom 96 well plate (use only inner wells).
Add 5 ul of DR1pep-SAPE oligomer reagent (tetramer) to appropriate wells.
Add 200 ul RPMI or PBS to the outer wells of the plate.
Place plate in CO2 incubator for 3 hours.
Chill plate on ice 5 min.
Prepare cocktail of desired secondary antibodies (I typically use 1 or 2 ul of each 2ndary per well) and dilute with media, RPMI or PBS so that you have 5 ul per sample.
Add 5 ul of 2ndary Ab cocktail to each sample. Pipet mix.
Let sit on ice > 20 min
Add 200 ul cold FACS buffer (PBS, 1% BSA or FBS, 0.02% NaAzide).
Spin plate in cold centrifuge 5 min, 1500 rpm

Aspirate with pipet tip or flick plate into sink.
Add 200 ul cold FACS buffer
Spin
Aspirate
Resuspend in either FACS buffer or PBS/1% paraformaldehyde.
Take to the FACS and count!

Notes: Remember to include plenty of controls including single-stained cells for compensation adjustment, control tetramers, control cells. Try to keep samples cold after the tet-stain incubation (3 hr) is over. Anti-CD3 antibody can stimulate the cells at RT or 37C and do weird things (downregulate TCR, maybe CD4, etc.)

Staining $CD4^+$ T cells with oligomers of class II MHCs

-tom

Prologue:

We have limited experience in this arena, but I think that is true for everyone in the field. Every cell/cell line seems to behave differently. What I detail below is what I generally recommend.

> *In my experience, some cells will stain while on ice, while others require elevated temperatures (for which 37° C is preferred). In my hands, the "4° C stainers" stain more brightly at 37° C, so I always recommend staining at 37° C. You might try both, if you have time, reagent, and cells. The advantage of elevated temperature is that reagent is internalized by the cell and subsequently trapped inside while receptors might recycle and gather more reagent. Thus, I find that longer incubation times yield brighter staining (although > 5 hrs seems to be overkill). Higher concentrations of tetramer also seem to give brighter stainings, but in the interest of saving this valuable reagent I perform most experiments between 10 and 50 micrograms/ml. For further discussion and examples of temperature dependent effects, see Cameron et al, J. Immunol Methods, 2002, MHC tetramers special issue.*

Importantly, be careful which oligomerization reagent you buy. I find that per microgram of DR, R-phycoerythrin conjugated streptavidin (SA-PE) from BioSource (lot 1101 or 1301 or 1401) gives the brightest staining (also note that both the Kappler and Kwok labs use this same reagent). Reagents from other companies are either less bright or almost completely ineffectual. I believe that this is a function of oligomericity. SA-PE conjugates are probably never one-to-one SA to PE. They are made by amine-amine crosslinking which can yield many different products depending on the ratio of reagents and extent of crosslinking. The SA-PE from BioSource appears to have an average MW of 15,000,000 Da (measured by dynamic light scattering). Thus, I believe this reagent is likely to be of higher valency than four. Reagents from other companies have probably been optimized for bright staining of biotinylated antibodies, for which a conjugate of multiple PE with low biotin-binding valency is optimal. For "tetramer" staining, a balance of high oligomericity and high PE content is desirable.

Reagents:

Biotinylated class II MHC with peptide of choice
(in our lab this is usually DR1 folded from inclusion bodies of alpha and beta subunits produced in E. coli and chemically biotinylated using maleimide-PEO-biotin).
Biotinylated class II MHC with control peptide
SA-PE from BioSource, Inc (lot 1101 or 1301 or 1401)
Cells, rested 7-14 days
RPMI
Extra media
cold FACS buffer (we use PBS, 1% BSA, 0.1% NaAzide, 0.5 mM EDTA).
FACS tubes and a FACS machine.

Oligomerize:

Oligomerized reagent is stable for several weeks. Eventually it will start to precipitate. I usually store at 4º C, although it can be frozen. Assay your MHC for biotinylation efficiency. Consider unbiotinylated MHC useless and use the concentration of biotinylated MHC for all further calculations. I advise adding the SAPE stepwise to maximize the formation of high order oligomers.

Aliquot desired amount of biotinylated MHC to tube. Add 0.25 µl of SA-PE per µg of MHC and mix by pipette. Wait several minutes. Repeat three more times (until you have added 1 µl SAPE per µg of MHC. (I determined this ratio empirically for my tube of SAPE and my cells. You may want to try a titration yourself). Add required volume of RPMI for a final concentration of 60 µg/ml (where half is MHC and we assume SAPE is 1 mg/ml. ie solution is 30 µg/ml MHC and 30 µg/ml SAPE).

Staining:

Prepare cells in a small volume of media. Aliquot 10 µl cells per well of a round bottom 96 well plate, or an eppendorf tube, or a PCR plate. Use any number of cells that you desire. I have used from 500,000 cells to 5,000 cells in 10 µl and seen no difference in the staining. Add 5 µl of MHC oligomer reagent. Seal wells/tubes with tape to minimize evaporation (or use some other trick to minimize evaporation). Place in a 37º C incubator 2-5 hours in the dark.

Chill on ice. Add secondary antibodies (CD3, CD4?) and proceed with staining as normal except with special attention to keeping cells cold continuously.

I recommend washing the cells in a 96 well plate, resuspending in a small final volume (30-50 µl) and transferring to 1.2 ml microdilution tubes (USA Scientific, Cat # 1412-0400). These tubes can be inserted inside FACS tubes for easy analysis.

Count them on the FACS, gate for live CD4+ cells. I typically find staining intensities of ag-specific tetramers to be 50- to 500-fold brighter than non-specific tetramers.

contact us:

Tom Cameron, lab of Dr. Larry Stern, MIT, Oct 2000.
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Thawing and Stimulating T-cell Clones

-procedure from Sadegh-Nasseri lab for passage of HA1.7 and Cl-1

T-cell Media
500 ml RPMI
5% human serum (heat inactivated)
5% fetal calf serum (heat inactivated)
5 ml HEPES pH 7.0
5 ml L-glutamine
2.5 ml Penn/Strep
filter and store at 4°C, good for approx. 1 month

B-cell Media
500 ml RPMI
10% fetal calf serum
5 ml L-glutamine
2.5 ml Penn/Strep
filter and store at 4°C

1. Thaw T-cells and put in 10 ml T cell media. Spin at 1500 rpm for 7 minutes.
2. Resuspend in T-cell media and count. Need 7 x 10^5-10^6 cells/well of 24 well plate. Keep cells on ice.
3. Count HLA-matched EBV-immortalized B cells. Need 1-2 x 10^6 cells/well. Spin down appropriate volume of cells and resuspend in 5 ml of RPMI.
4. Add appropriate concentration of sterile peptide (1μM of HA peptide for Clone-1 and HA1.7) and incubate at 37°C for 1-2 hours.
5. Spin peptide-pulsed EBV-B cells at 1500 rpm for 5-10 minutes and resuspend in T cell media at 1-2 x 10^6/ml.
6. Thaw feeder cells (PBLs) from 3 different donors. Need 1-2 x 10^6 feeders/well. Thaw into 20 ml RPMI. Spin down at 1500 for 5-10 minutes and resuspend in T cell media.
7. Irradiate cells:
   PBL= 5,000 rads
   EBV= 10,000 rads
8. Spin down PBLs and EBVs and resuspend in T-cell media such that EBVs are at 1-2 x 10^6/ml and PBLs are at 1-2 x 10^6/100 microliters.
9. Set up wells of 24 well plate with Tcells (7 x 10^5- 10^6/well), EBVs (1-2 x 10^6/well), and fedder PBLs (10^6/well) and bring up to a final volume of ~2 ml with T cell media.
10. Put plate at 37°C overnight.
11. Supplement with 20 Cetus units (120 IU)/ml of IL-2.
12. Leave at 37°C for approximately 7 days or until T cells look like they are in resting stage (round not elongated). If T cells look crowded (probably at day 4 or 5), split wells in half adding 1 ml of T cell media without IL-2 to each well to bring volume back up to 2 ml/well. Once T cells are resting they can be used in an assay, frozen, or restimulated to expand.

PBMC prep:

-Tom

Obtain fresh blood, preferably that was NaHeparin treated (green topped vacutainers). (K3EDTA is okay too, I think). Dilute with an equal volume of room-temp RPMI. (You can leave diluted blood on your bench overnight if necessary and the cells will be pretty healthy the next day. Add some Gln if you do this.) Aliquot 10-20 ml of Ficoll-Paque Plus (Amersham-Pharmacia 17-1440-02) into 50 ml conicals, and then layer on (slowly) diluted blood. Spin 1500 rpm in swinging bucket rotor 20-40 minutes at RT, and stop without brakes. Aspirate away most of the serum layer, and then collect the buffy coat, trying to minimize the ficoll while maximizing cells. Pool buffy coat from various tubes, wash a couple times with RPMI or PBS. If you want to freeze them, resuspend in 50% serum, 50% RPMI, chill, add DMSO to final 8 or 10%, aliquot into cryovials. I typically freeze aliquots of about 10 million PBMCs. Note that you can't use the same Ficoll for human cells as for murine ones.

Complete Medium: RPMI 1640 supplemented with Glutamine, Pen/Strep, 10 mM Hepes pH 7, 7-10% Human Serum, male AB, heat inactivated 25 min at 56C (I have recently been using serum from Sigma and it has been working fine).

T cell Proliferation Assay:

-Tom

Aliquot PBMCs or T cells into TC-treated round bottom 96 well plates. Add additionally EBVs that have been irradiated 2 hrs at the cancer center irradiator along with stimulus of interest: peptide (try from 0.1 mM to 20 mM, typically), control peptide (TfR?), Positive control (PHA 2 ug/ml, IL-2 40 u/ml: PHA, sold as PHA-P from Sigma, freeze stock at -70C, use thaw for 2 weeks when stored at 4C, and IL-2 from Chiron sold as Aldesleukin for clinical use). Final volume should be between 100 and 150 ul. Do wells in triplicate. Either two or three days later (or maybe wait longer if response is small) add 1 uCi of 3H-Thy (NET-027X from NEN-Perkin Elmer) to each well (dilute with RPMI and add 10 ul RPMI with multichannel). I usually resuspend the cells with the multichannel to ensure even distribution of the 3H-Thy in the solution. After 8-24 hours, collect the plate on our harvester onto glass fiber filter mat, dry it, add scintillation fluid, and count it (1 min per well). I have found that it is very difficult to detect pep-specific proliferation for HA, FluB or TT in PBMCs from healthy patients. However, even though you may not see a proliferative response, the cells may grow up during an in vitro stimulation. Consider adding the 3H only on day 4 or 5 to try and detect the cells more sensitively.

CFSE labeling:

-Tom

CFSE (carboxy fluorescein diacetate succinimide ester, Mol. Probes C-1157) stock should be made in DMSO at 5 mM and stored at -70C. Each batch of CFSE should be titrated on cells to determine optimal staining. Note that if you stain too brightly, you won't be able to compensate the bleed through into the PE channel. In the Kwok lab they report labeling with 0.8 uM CFSE in PBS, 10 min 37C, quenching with FBS and washing with RPMI. In Lucy Wedderburn's lab they labeled with 5 uM CFSE in RPMI 10 min 37C, quenching with FBS and washing with RPMI. Since RPMI contains mM amounts of amines (amino acids!), labeling in PBS makes more sense. But in my hands, using CFSE that has been freshly made or thawed from -70C, it is WAY too bright at these concentrations. I suggest using 50-200 nM CFSE in RPMI. I don't know why it is so different in my hands than in theirs, unless their reagent is significantly less potent because it is prepared poorly (maybe not made fresh and stored at 4C? or RT?).

In Vitro Stimulation: -tom

For a long time, I didn't get good stimulations in vitro. I think there were several reasons: fresh cells are better than frozen, higher density is better than lower, higher pep concentration is better than lower, more IL2 is better than less.

Suggested protocol: Prepare fresh PBMCs, (optional: CFSE label), count them carefully, plate them at 4 million/well of a 24-well plate, or equivalent density (cells per surface area) in 48-well or 96-well flat bottom, or anywhere from 20,000-50,000 per well of a rnd-bottom 96-well. Add peptide (HA?) to 5 uM. Remember to do a well of control peptide (TfR?) and maybe to do a well (small well?) with + ctrl (PHA/IL2?). On Day 5, add 20-40 units/ml IL-2. Continue adding IL-2 every 3-4 days at 40 u/ml. Be sure you use good IL-2 (I like the stuff from Chiron, store it at -70C long term, and used thawed aliquots in < 1 week). By day 6/7 you should have significant cell growth, probably between 0.1% and 3%, and I think the cells will keep growing for several more days so long as you are adding IL-2. I haven't studied this directly. I think if you keep on adding IL-2, eventually the non-dividing cells die off (i.e. become > 50% specific). It is an open question as to whether all the dividing cells are ag-specific or not.

However, you can get this to work with frozen cells, or at lower densities, or at lower peptide concentrations. In particular, it is possible that pep concentration will have interesting effects on the types of cells that get stimulated and the extent of their stimulation. Kappler and Marrack have a PNAS paper reporting that in mice, lower doses of ag lead to proliferation of higher affinity clones. A titration of peptide might be an interesting experiment in the human system as well.

Further Culturing and Sorting:

During the first few stimulations "out of the arm" the T cells will grow like mad. CFSE shows that in the first week, the tet+ cells have divided at least 6 or 7 times. Abby Meyer in Bridgette Huber's lab sorted OspA specific cells with DR4-OspA tets directly from PBMCs and then stimulated with PHA/IL2. Within two weeks, for many clones the single cell per well had grown to a sizable plug of > 10,000 cells! It seems like each round of stimulation diminishes the proliferative potential of the cells. So: if you want to do any cloning, do it as early as possible or else they will never grow up.

A.Meyer/B.Huber/D. Haffler Cloning Procedure: Sort one cell per well onto rnd bottom 96 well plates that have 50-100,000 allogeneic irradiated PBMCs. Add PHA to 2 ug/ml. Next day add IL-2 to 40 u/ml. Every 3 or 4 days, remove half the media and replace with new, pre-warmed media with fresh IL-2. Two weeks afterwards, they see plugs for clones. I think that if you follow this procedure having sorted cells that weren't fresh PBMCs, you may need to restimulate the wells with irradiated allogeneic PBMCs/PHA/IL2 to get sufficient cell growth to see a pellet. They suggest stimulating cells this way every two weeks, always stimulating in rnd bottom 96 well plates, and transfering to flat bottom for when the cells get dense, during rounds of IL-2 addition.

My thoughts on this: I'm not sure that culturing with PHA is better than with HA. Some cells might respond better to PHA or to HA. The choice is yours. In my experience, I get better stimulation from HA, but I haven't ever done this on the single-cell cloning level, and bulk populations probably get skewed by either approach. But I think they're right that the cells like to be stimulated in rnd bottom wells. Unfortunately, this gets cumbersome when you have a lot of cells.

Routine passage of lines is usually about 1 million T cells per well of 24 well plate plus 1 million irradiated (2 hrs) EBVs and 1 million irradiated (1 hr) allogeneic PBMCs (newly thawed) plus appropriate peptide. Sometimes I stimulate denser, and split the next day or soon afterwards. Day after stimulation, add IL-2 to 40 u/ml, and again every 3/4 days. If the media gets too yellow, either split 1:2 into new wells, or remove 1/2 the media and replace with fresh pre-warmed media. If the cells aren't growing well, try stimulating in rnd bottom wells, about 20-40,000 T cells per well.

In Vitro Stimulation of PBMCs for subsequent Tet staining:

Tom Cameron, Feb 2001.
This is what has worked for me.

Prepare PBMCs.
Optional: label with CFSE.
Put 5 million per well of a flat-bottomed 24-well plate in complete media.
Add antigen at a moderate dose (about 1-10 uM?) and mix by pipet briefly.
If you have enough cells, use at least one well for stim with 5 uM HA as a pos. control.
Use at least one well as a negative control with either control antigen or no antigen.
Day 6 or 7, add IL-2 to 50 u/ml
3-4 days later add IL-2 to 50 u/ml. If media is turning yellow, remove and replace half with prewarmed fresh media.
Continue adding fresh IL-2 every 3-4 days.

You can try a tet stain at any point. In my experience with the HA peptide, I see approx. 1-3% specific cells at day 7, and 20-60% specific cells at day 14.

If you're sample is an HIV+ sample, you will probably need to add anti-retrovirals.

HA is an excellent antigen for most people. It is as good a postive control as you will get.

Anergization of T cells

Protocol based on experiment of April 9th

Reagents:

T cells and EBVs
Peptides, OKT3, PHA, IL2, PMA, Ionomycin (all sterile)
Tissue culture plates and 15 ml conical tubes

Day 1:

Plan your experiment and calculate the number of wells and cells you will need.
Plan triplicate samples of the proliferation.
Coat wells of a flt bottom 96 well plate as desired with OKT3:
dilute OKT3 with PBS or RPMI to 5 ug/ml, and put in appropriate wells
incubate at Room Temperature 2 hrs.
Wash wells several times with PBS or RPMI. Don't allow to dry.
Count T cells and plate them 200,000/well (150 ul/wl) in flat bottom 96 well plate
Add "Anergizing Stimulus": Control Peptide, Specific peptide (> 1 uM).
Leave 24 hrs in 7% CO2 incubator.

Day 2:

Collect healthy EBV 1.24 (DR1+) cells in T cell media and irradiate 10k rads.
Warm some T cell media.
Collect the "anergized" cells into 15 ml conical. Dilute with RPMI to 10 ml, centrifuge, aspirate (to about 50-100 ul; don't suck up the pellet!) and resuspend in small volume of T cell media.
Add EBVs and media to each "anergized" sample so that you have 250,000 T cells/ml, 250,000 EBVs/ml, and enough volume to plate all the wells that you will need (and a little extra, of course).
Aliquot samples 100 ul per well into rnd-bottom 96 well plates
In extra space of the plate, aliquot EBVs with no T cells at the same density.
In a separate flat-bottom 96 well plate, prepare the "proliferation stimulus" samples: In whatever manner you prefer, obtain sufficient T cell media containing appropriate T cell stimulus (peptide, PHA/IL2, or PMA/Ionomycin) at 3x final concentration.
Aliquot 50 ul of "proliferation stimulus" samples onto the 100 ul of cells. Pipet mix briefly.
Leave 48 hrs in 7% CO2 incubator

Day 4:

In a sterile multi-channel trough, dispense (20 ul * # of wells) of T cell media.
Mix with (# of wells) uCi of 3H Thymidine. Mix thoroughly (and carefully).
Dispense 15 ul per well of proliferation plates (including the EBV alone wells).
Pipet mix as you go along.
Leave 15 hrs in 7% CO2 incubator

Day 5:

Collect plates onto glass filter mats using plate washer.
Put in scintillation fluid.
Count 1 min/well.

Calcium Release Assay on the Fluorometer

3/11/98
Work in Progress
-Tom

So far the DM3000 program isn't working yet. It is specifically designed for cation measurement by alternating between 400 and 500 nm and plotting the ratio. This handout details performing the experiment solely by monitoring emission at 400 nm. This gives a pretty good indication of whether a stimulus triggers or not, but we really need to obtain some sort of normalized response (free vs. bound Indo) to compare different cell preps on different days. This can be done some manipulations at the end of the experiment (see *optional* section) or by looking at the 400/500 ratio.

Cuvette:

It is hard to find a good cuvette for the job. Needs to be small volume, quartz (to pass ex 338 nm light), and easily stirred. I have been using **29F-Q-10-MS** (nominal volume 1.4 ml) which means stoppered semi-micro (29) fluorimeter (F) quartz (Q) 10 mm pathlength (10) stirring (MS). The cuvette has a rectangular chamber of approx 3 mm by 10 mm and broadens near the bottom to allow a stir bar to rotate. In this cuvette, it is ok to **use 0.65 ml the 14 mm spacer** and collect the emmision through the etched glass near the bottom of the cuvette. I think I should probably purchase 23-5.45-Q-5 (square micro, 5 mm ID, 45 mm height, quartz, nominal volume 0.875 ml) and FCA5 (adapter) and use with 2mmx5mm stir bars (shaved slightly). I could probably cut down to 250-300 ul in this cuvette.

Machine Start-up:

be sure to fire the lamp when no other electronics are on. Let the lamp warm up for > 15 min before using.
Run Instrument Control Center program.
If desired, run an excitation (em: 400, ex: 300-380) or emmision (ex: 338, em: 350-550) scan.
For a typical time course calcium release experiment use ex:338 slit 1.5, em:400 slit 8, collect a point every 2 seconds, time average for 1.5 secs, set temp to be 37C, and stir between 5 and 10. The slits are relatively arbitrary. I chose these slits to maximize signal while minimizing bleaching and not saturating the detector.

Sample Prep:

Dissolve Indo-1 at 1 mM with DMSO.

Load cells with 4 uM fresh Indo-1 (in normal media) for 30 min at 37 C. To save Indo-1 I have been loading cells at high density (about 10E7 cells/ml) (this requires spinning cells down once and resuspending in smaller volume). After they are loaded, spin once, suck off supernatant, and resuspend cells in Buffered Salt Solution (BSS: 135 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 5.6 mM Glucose, 20 mM Hepes pH 7.4, store sterile at 4C) with 0.1% gelatin (to make BSS/Gel). Store cells on ice until ready to use (seems ok for several hours).

Experiment:

Warm some BSS/Gel to 37C. Dilute Indo-loaded cells into warm BSS/Gel to 5E5 cells/ml in cuvette. Run time-course experiment baseline for 5 minutes (be sure stirring!). I very often see a downslope for about 4 min and then a gradual up slope. In any case, these ought to be relatively small drifts compared to the expected signal. After five minutes of baseline, turn off the computer room light and either open door or turn on xray room light. Lift top of sample chamber and quickly inject your stimulus (be sure to pipet into the solution, not onto the cuvette). Close the sample chamber lid. Watch the absorbance. You should see em at 400 increase significantly within 30 seconds and peak sometime within the next five minutes. Collect data points for at least 10 min after stimulation.

Optional: add triton to 0.25% to lyse cells and see 100% calcium signal. Then add EGTA to 10-15 mM to see 0% calcium signal (be aware of dilution effect!).

Note: Why Gelatin? Identical experiments using 15 ug of Jovi-1 showed a slower and smaller response without gelatin than with. So I believe that it is acting as a carrier protein, binding to nonspecific sites on the cuvette walls so that our protein won't. Gelatin is probably better than BSA because BSA is notoriously "sticky" and may bind peptide or MHC.

Calculation:

$$I(t) = (F_s(t) - F_b)/(F_{max}-F_{min}-F_{out})$$

where I(t) is the fractional Ca-bound Indo response, $F_s(t)$ is the total fluorescence of sample after addition of stimulus, F_b is the baseline fluorescence of the cells before addition of stimulus, F_{max} is the measured fluorescence after addn of 0.25% Triton (100% bound), F_{min} is the measured fluorescence after addn of EGTA (0% bound), F_{out} is the fluorescence measured from a different sample of cells without stimulus minus their fluorescence after addn of EGTA (without triton) (F_{out} ends up representing the fluorescence due to extracellular Indo-Ca complex. (F_{max}-F_{min}-F_{out}) represents the maximal indo-1 fluorescence within the cell. ($F_s(t)$ - F_b) represents the stimulated Ca-Indo response.

Intracellular Cytokine Staining Protocol:

written by Tom, synthesized from various protocols found at Sigma, BD, Caltag, Zoran, and on the web.

Reagents:

Stimulus: irradiated EBVs, peptide, PMA/Iono, PHA/IL2 or whatever you want.
BFA: Brefeldin A, Sigma B7651. Make stock 5 mg/ml in DMSO
Freeze at -20C or -80 in 20 ul aliquots. Use at a final conc. 10 ug/ml.
Fixing Solution: 4% PFA in PBS, stored cold and < 1 month old
Make with dry paraformaldehyde in PBS at 56-66C stirring for 30-60 min
Cool down, adjust pH to 7.2-7.4, filter.
Permeabilizing Solution: PBS, 1% FBS, 0.1% NaZ, 0.1% Saponin (Sigma S-4521), adjust pH, filter, store cold.
FACS Buffer: PBS, 1% FBS, 0.1% NaZ.
FACS Buffer with EDTA: PBS, 1% FBS, 0.1% NaZ, 5 mM EDTA.
Anti-cytokine mAb, Fitc or PE conj:
Use Sigma Aldrich P-6847, anti-hu-IL2-PE, use at 1:20 dln
Isotype control mAb, Fitc or PE conj
Use IgG1-PE Isotype Control, at a final 5 ug/ml.
CD4 mAb, with a different fluor
Diatec CD4-APC Cat # 3023

Protocol:

·Wash cells, resuspend in T cell Media, and resuspend with *stimulus* in rnd-bottom 96 well plate. (max 100,000 cells/well?) Incubate at 37C with CO2 for 2-3 hours.
·Add *BFA* to 10 ug/ml in each sample and resuspend to mix. Incubate 37C with CO2 for additional 4 hours.
·Spin down (5', 1500 rpm), flick, resuspend in 40 ul *FACS buffer with EDTA* containing *CD4-APC* (1:20 dilution). Incubate RT, 15 min.
·Add 160 ul *Fixing Solution*. Incubate RT, 15 min.
·Spin Down, flick, resuspend in 200 ul *FACS buffer*
·Spin Down, flick and resuspend in 40 ul *Permeabilization Buffer*. Split samples, and to one set add *anti-Cytokine mAb*, and to the other add the *isotype control*. Incubate 4C 30 min.
·Add *Perm Buffer* to 200 ul, spin down, flick, resuspend in 200 ul *Perm Buffer*, spin down, flick, resuspend in 30-80 ul *FACS buffer*.
·Analyze on FACSCalibur Flow Cytometer.

Also consider using a positive control, maybe PMA/Iono or PHA/IL2. Note: I have found PMA/Iono leads to large scale CD4 downregulation.

S2 cell culturing and transfection

Tom 7/15/1999

Cloning:

Clone into pRMHA-3 vector (See Nucleic Acids Research, Bunch, Grinblat and Goldstein, Vol 16, No. 3, 1988, pp1043). Include native Signal Sequence of your protein. I have seen successful constructs using either EcoRI or BamHI as the upstream site. Purify construct via Qiagen column and after Isopropanol ppt and EtOH wash take up with sterile TE and move to a sterile eppendorf tube. Also purify some pNeo vector (or you could get the hygromycin selection vector that Invitrogen sells).

General tips on growing S2 cells:

They are also called Schneider cells. They are a Drosophila melanogaster cell line (Available from ATCC, CRL-1963). I read somewhere that they are of macrophage lineage, but don't quote me on that.

Freeze 10 million/ml in 1 ml aliquots of 10% DMSO, 90% Media (w/ Serum). Thaw like any other cell line. They like to be dense!! Serum Media: I use S2 media from Sigma, supplement with 10% FBS, with any combination of the additives Pen Strep Gln Fungizone Gentamicin (usually PS or Gent, not both). It sounds like they grow well in any insect-cell serum-free media on the market (I've heard about Ex-cell, BaculoGold, Sf900 all being used with success), but I've only used Sf900 (with PSFQ). Adaptation seems to be pretty easy. Pass from 100% to 50% to 25% to 10% to 2% to 0%, or something like that. In serum media, they adhere to plastic very loosely and can be knocked of by banging the flask. In SFM they adhere pretty tight, but they can mostly be removed by pipetting. They seem to double every 24 hrs in serum media, and about every 30 hrs in SFM (or maybe a little faster). In order to pass from plastic into spinners, it is best to let the cells overgrow two T-75 or one T-150 flask so that there are a lot not sticking to the plastic. Pipet vigorously to remove most cells from the plastic and transfer to a 100 ml spinner flask. So far as I can tell, the cells like to be denser than 0.5 million/ml always, plastic or spinners. In spinners, they grow happily up to 10 million/ml and will start to saturate but still reach up to 20 million/ml. They are smaller than Sf9 cells.

Transfection:

Buy Gibco CaPi kit (18306-019). Mostly follow their protocol. Briefly, here is how I suggest. Plate 3 million cells per well into 6 well plates. Leave overnight. Somehow sterilize your DNA. (Use sterile Z-spins if you want with the transfection water before you add Ca Sln). Mix 0.25 ug pNeo with 5 ug of each plasmid (alpha chain and beta chain). As according to Gibco directions (in TC hood), mix into water to 215 ul total, add 5 ul Ca Sln, add 25 ul more Ca Sln. Make 1X HBS 0.25 ml per transfection (dilute 10X HBS to 1X and add 15 ul NaOH per ml HBS). In a 15 ml conical mix 0.25 HBS with 5 ul Pi sln. While vortexing (in TC hood) this solution drip in plasmid/Ca soln. Let sit RT 30 min. Add dropwise to cells while gently rocking. Store RT 12-24 hours. Transfer cells to 15 ml conicals, wash with media once, also rinse out wells with media or PBS. Replate into original wells. Either add CuSO4 or wait another day and then add 1.5 mg/ml G418. I have heard that Lipofectin can be used for higher efficiency

transformation, especially with some trick about plating the cells in 90% SFM the night before (?).

Handling of lines:
After washing the cells, replate into the same wells with new media. Put the transients into media with 0.5 mM CuSO4 (sterile, of course). 1 mM might be better but you should test it to see. Put the stables into media with 1.5 mg/ml active G418 (Geneticin) two days after transfection. Carry the stables in 1.5 mg/ml G418 in the same wells for three weeks. At the end of the first week, spin down the plates and replace media with new media/G418. Then pass the cells something like 1:1 or 1:2 every three/four days for the remaining two weeks, still keeping them in the same wells. At the end of three weeks, start to expand the lines to flasks and spinners but maintain the G418 until you get to a 100 ml spinner. Then make 5-10 freezes of cells, with 10-30 million cells per vial. At this point you can probably start being lazy with G418. It is probably good to continue to use G418 in small scale culturing but it should be unnecessary while scaling up.

Single Cell Cloning:
I have a procedure from D. Zaller on plating the cells with irradiated cells and overlaying with soft agar and looking for colonies. It is probably easy, but I decided to do limiting dilution instead. The problem with limiting dilution is that it takes about 3-4 weeks for untransfected S2 cells to die in the presence of G418. Briefly, dilute untransfected S2 cells to 0.3 million / ml and dope in transfected cells to get either 1.3 or 0.3 cells per 200 ul. Aliquot 200 ul per well into flat bottom 96 well plates. Store in a humidified environment at RT (in a tupperware container with holes poked in the top or in a pipet-tip box with a wet paper towel). Each week, spin plates 5 min 1000 rpm and aspirate and replace media. It is probably best to use media with serum.

Dennis Zaller's Single Cell Cloning in Soft-agar:
Irradiate S2 wells with 10,000R. Use 3 petri dishes per cell line, 2 million irradiated cells per petri dish. Wash irradiated cells with SFM, resuspend at 1 million/ml in SFM, seed 2 ml onto 60 mm petri dish and let them settle 1-3 hours. Prepare overlay media: Mix 60 ml Sigma S2 media with 20 ml FBS and 10 ml 2X Sigma S2 media, warm to 37C, add 10 ml melted 5% bacto-agar, mix and keep warm. Gently aspirate media from petri dishes and replace with 5 ml overlay media and incubate 10 min on a level bench at 4C, then move to RT and leave overnight. Next day, prepare overlay media again but with 0.36% final agar concentration, 1 ml per petri dish. Also prepare warm S2 media with 20% FBS, 0.2 ml per petri dish. Count cells and prepare, for each line, a tube with 0.2 ml S2 media/20% FBS with 6000, 2000, 667 cells. Add 1 ml of overlay media and then overlay onto yesterday's feeder-plates. Cool 10 min 4C and then incubate humidified RT for 10-16 days. Microscopic colonies should be visible after 3 days, but discreet colonies should be visible at 10-16 days. Pick colonies using micropipet tips and a dark field plate microscope, transfer to wells of 96 well plate with 100 ul media with 20% FBS. Slowly expand cell line and test for expression. Probably somewhere in here we need G418!!!

Large Scale Culturing:
Cells grow fine in 100 ml and 1 L spinners. Spin as fast as you can while avoiding excessive foaming. Use only 500 ml in a 1 L spinner. I keep the cells > 0.5 and <5 million/ml until I'm ready to induce. They really like air! But they'll grow really dense! I have seen various reports of other culturing conditions that might be easier for large scale but I haven't tested any. Roller bottles will work but you need to rotate them really fast to get enough air and it ends up getting expensive. I think a 6 L spinner with air bubbling should be okay. Baffled flasks on a rotating platform at 100-200 rpm is reported to work also, but be sure to keep a low volume per flask (500 ml in a 2 L).

Purifying Protein:
Collect sup after six days (or use a time course to optimize for some other time) and concentrate 20 fold, add protease inhibitors (skip PMSF and IAA if you are making DR with free cys) and EDTA and Azide. If viscous, add MgCl2 and Dnase and stir 10 min RT. Flow over Protein-A pre column and then Ab column for purification. Elute with Glycine pH 3 or Caps 11.5. Note: do NOT exchange into PBS while concentrating. There is something in the serum free media that stabilizes empty DR protein.

Example transfection procedure:

Day 1:
Four different transfections in four wells of a 6 well plate using CaPi Gibco kit:
1) 10 ug IAsAlpha-1 10 ug IAsBetaCys-1 with 0.5 ug pNeo
2) 10 ug IAsAlpha-2 10 ug IAsBetaCys-2 with 0.5 ug pNeo
3) 10 ug IAsAlpha-1 with 0.5 ug pNeo with carrier DNA
4) 10 ug IAsBetaCys-1 with 0.5 ug pNeo with carrier DNA

Day 2:
Wash cells in a 15 ml conical and return them to original wells without antibiotic. Remove 400 ul of cells for transient expression test and add antibiotic to the remainder.

For Transient Expn Test: with the 400 ul of cells.
Day 2: In a flat bottom 96 well plate, put 200 ul into first well and 100 ul into the next two wells. Add 100 ul media (no antibiotic) to wells 2 and 3. Add CuSO4 to the first well.
Day 3: add CuSO4 to well 2 and split well 3 1:1 into well 4.
Day 4: add CuSO4 to well 3 and split well 4 1:1 into well 5.
Day 5: add CuSO4 into well 4 and split well 5 1:1 into well 6.
Day 6: add CuSO4 to well 5 and split well 6 1:1 into well 7.
Day 7: add CuSO4 to well 6.
Day 8: test expression

For Stable lines: grow cells in 1.5 mg/ml G418 as I detail in my methods sheet. If you want, I think you could set up limiting dilution plates at this point (only one day after transfection) but it may not be worth the effort yet. Maybe it would be better to wait a week or two before you do this.

Transfection of S2 cells with Lipofectin:

Tom Cameron, June 1, 2000
Modified from the Gibco directions for Lipofectin, Cat. No.: 18292-011

Do all this work sterile in the TC hood.

Previously: Thaw and grow untransfected S2 cells in media with Serum until happy!

Day 1:

Plate S2 cells at 1 million/ml in desired plates in 90% Sf900 PSFQ, 10% Media with serum and PSFQ (so final 1% serum).

Day 2:

Prepare Solution B: Dilute Lipofection Reagent 1to5 with Sf900 (see final volume listed below) and allow to stand at Room Temp 30-45 minutes.
Sterilize your DNA using a Z-spin sterile centrifuge filter.
Prepare Solution A: Dilute DRalpha, DRbeta and pNeo DNA into Sf900 (see amt of DNA and final volume listed below).
Combine Sln A with Sln B and incubate at Room Temp for 15 minutes.
Meanwhile, spin cell plates 1500xrpm, 5 min, aspirate media, add Sf900, swirl, spin plate again, aspirate media, add appropriate volume of Sf900 and swirl.
Add SlnAB to cells and swirl vigorously or pipet mix.
Leave overnight.

Day 3:

Spin cell plates 1500xrpm, 5 min, aspirate media, replace with Typical Culture Volume of Media with 10% Serum and PSFQ

Day 4 or 5:

Either add CuSO4 to final 0.5 mM *OR* add media with G418 (Geneticin antibiotic) to final concentration 1.5 mg/ml (calculated for active antibiotic).

Day 8-10:

Either sample sup for protein production *OR* spin plates and replace media with fresh Media with 10% serum and PSFQ ang 1.5 mg/ml G418.

Further Culturing:

If a lot of the cells died, then you will have to passage the cells as in "Day 8-10" without splitting the cells for a while longer. However, if most of the cells survived and they are growing very dense and happy, I suggest on day 13-16 split 1 to 3 (you could either discard the extra cells or put in a different plate and induce them for a test expression) with Media with 10% serum and PSFQ/G418 and starting on approx day 18-20, start expanding the cells to larger volumes (with serum media). Once the cells have been in culture for four weeks, make some freezes! (at least three of 10 million each, but more if you can). Then do some test protein expressions!!! After four weeks the line should be stable and

fully G418 resistant. After you have made plenty of freezes, you can start to passage the cells into Sf900 and to stop using G418.

Scale Options:

	Typical Culture Vol. (ml)	Day 1 Cell Density (million/ml)	DRalpha DNA (ug)	DRbeta DNA (ug)	pNeo DNA (ug)	Sln A Vol. (ul)	Sln B Vol. (ul)	Day 2 Transfxn Vol. (ml)	Day 3 Culture Vol. (ml)
6well	3.00	1	3.00	3.00	0.20	100	100	2.00	3.00
12well	1.20	1	1.19	1.19	0.08	40	40	0.79	1.20
24well	0.65	1	0.63	0.63	0.04	21	21	0.42	0.65
96well	0.20	1	0.23	0.23	0.02	8	8	0.16	0.20

Notes on Peptide Synthesis:

By Tom Cameron, Stern Lab, Oct 24, 2000.

These notes are for synthesis of simple peptides on our ACT357 machine using fmoc chemistry. Some details are not included so feel free to ask people for advice (Tom, Jen Z., Jenny, Jen Stone, Tanyel …)

Getting Started, Useful Software:

Choose peptides to synthesize. Double check sequence!

For DR1-binding epitopes, we have some prediction algorithms programs for which we have posted on the Stern Lab website under "MHC tools and tips." My programs are called EpiPred and are in Microsoft Excel format. There should also be a copy of these spreadsheets on the CD copy of my dissertation.

Decide if you want N terminal acetyl or amine, C terminal acid or amide.

For C terminal acids, use a Wang resin with Cterminal AA pre-attached (make sure we've got it in stock). For Cterminal amide, you can use either a blank Rink resin or Tentagel S RAM resin and couple the C-terminal AA on the machine.

On Krispie there is a program called *Peptide Companion* (designed in the labs of Paul Matsudaira and Peter Kim at the Whitehead Inst.). Under its "Difficult Sequence" utility it will predict the synthetically difficult coupling reactions. A peptide with any Red dots, is "a little tough" and I suggest double coupling, 60 min each. A peptide with lots of Red dots probably won't work without fancy tricks that I don't know. (Consider having it made at the Biopolymers lab). A peptide without any Red dots is probably pretty easy and you could get away single or double couplings, 30 min each. *Peptide Companion* will also predict HPLC retention times, and calculate MW.

Go to "friedrice:/c:/people/Peptide Synthesis/AA usage", and open in MS Excel

Enter sequences and obtain # times each AA is used. For unusual AA enter X and calculate by hand.

Go to "friedrice:/c:/people/Peptide Synthesis/Peptide worksheet master" and enter required info to obtain weights and volumes of various reagents needed.
I suggest for easy peptides doing 1x coupling, 5-fold excess AA, HOBT/DIC with capping (DIPEA/Ac2O). For slightly harder peptides, couple twice. For much harder peptides, ask someone else (use TBTU?).

Reagent Prep/Machine Setup:

Use NMP and DMF that has been treated with the amine-removing drop-bags that Danny has always used. I don't know where to buy them if we run out!

Take out dry reagents (AA, resin, HOBT) from freezer to warm up before weighing. They are expensive! Warm them up first!

Wear gloves and goggles while dispensing solvents. Dissolve AA in 50 ml Ppro conicals or directly in AA bottles if small volumes. Dissolve DIC, HOBT, DIPEA, Ac2O directly into reagent bottles.

Some of the AA don't dissolve easily (esp. Asn and Gln). Vortex and bath-sonicate. If you need, add a little extra solvent (slightly lower concentration won't kill you).

Put all AA solutions in bottles in rack in machine. Put lid on.

Top up DMF bottle (be sure to use DMF that has one of the amine-removal drop-bags), and top up 25% piperidine. Make sure there is some fluid in the third bottle also (DCM?) or else the machine won't work properly.
Empty the waste if not already empty.
Dispense resin into appropriate wells (far left well is 1, far right is six, closer right is 36).
Machine setup checklist:

- Plenty of DMF? (start with a lot!)
- Plenty of Piperidine? (start with 4L)
- Some fluid in bottle 3?
- HOBT, DIC, DIPEA, Ac2O?
- AA?
- Are all HOBT, DIC, DIPEA, AC2O and AA weighed down by donut or rack-top?
- Is the reaction block secured?
- Waste empty?
- Vacuum for waste turned on?

Program setup:

Run ACT357 from desktop
Check system setup:

- Under System Setup/AA settings check bottle assignments, crosscheck with machine setup.
- Under System Setup/Reagent settings check bottle assignments, crosscheck with machine setup.
- Under System Setup/System fluids check bottle assignments, crosscheck with machine setup.
- Under Utilities/Calibration/Rack Calibration/ check that AA and Reagent diameters are set appropriately (22 and 90 mm for large scale, 11.5 and 14 for small scale (ie with special inserts in AA/reagent bottles)),

Enter peptide sequence:

- Go to System Setup/Sequence Builder
  Start with a Global delete (Shift-delete) to clear all old sequences (esp. if they are in bottom wells and you might not see them at first glance!).
- Enter sequence. Remember to omit C-terminal AA if you already have that AA on resin!
- Double check sequence.

Examine and choose reaction protocols:

- Go to System Setup/Protocol Design/Protocol Design.
- Look at the various protocols and choose one that is close to what you want.
  I suggest something like two Piperidine deprotection steps, washes, single or double coupling of AA for between 30 to 60 min while shaking, washes, capping with DIPEA/Ac2O once, washes.
- Adjust volumes to suit your needs.
  - Make sure moles HOBT = moles DIC >= moles AA in each coupling reaction.
  - Make sure total volume of any one step is not too large (<= 5 ml? I'm not sure max).
  - Make sure volume of HOBT, DIC, AA, DIPEA, Ac2O dispensed matched what you planned for on your Excel worksheet.

Save protocol and remember its name.

Think about the last coupling step and decide strategy

Do you want Nterminal fmoc removed? Acetylated? Biotinylate or FITC label?

Examine protocols, choose appropriately, and modify Peptide Sequence Builder as necessary.

Include extra washes at the very end. Consider a DCM/DMF wash (good idea).

Go to System Setup/Protocol Design/Protocol Selection and choose appropriate protocol for each coupling step. NOTE: if you have changed your protocol at all, you must re-select it at every appropriate position in order to "update" to program as to your changes. Otherwise it will run according to your old protocol! (Stupid program glitch!).

Double check Protocol Selection!

Note, you can only run a single protocol at a particular coupling step, but you can leave gaps in the sequence builder. So, for example, if you are synthesizing a 10mer and a 15mer, and want to remove the fmoc at the end of the sythesis from both, enter the 10mer in the first 10 cycles, the 15mer in the first 15 cycles, and in the 16^{th} cycle, put X for AA in each position, for both peptides, run a fmoc-removing protocol at the 16^{th} position, and your done!

You're almost ready to run! Just double check everything one last time.

Check that waste is empty

Check that vacuum is turned on

Check that all reagent/AA bottles are weighed down

Check that you put resin is in the appropriate wells, and there aren't any plugs where the computer doesn't expect them.

Turn on printer

Print out Peptide sequence from Sequence Builder (F7 I think)

Print out your protocols (F7 I think)

Funny program glitches to remember:

Double check Reagent/AA bottle diameter settings for large/small scale settings (rare but important).

If you change your protocol, re-select it!

Is there some fluid in every System fluid bottle?

If it doesn't seem to run properly, try exiting program and restarting Act357 from Windows or Dos.

Run it!

Go to Automatic Mode.

Enter Start Seq Pos 1, Seq Stopping Pos X (you decide!), Starting Step # 1, Starting Rxn vessel 1, Starting AA vessel 1, Remaining Repeats –1 (-1 means ALL).

Tell it to report to printer, NOT to interrupt in case of printer failure, and to flush system.

Start it! Woo Hoo!!!!!!

Watch it!

Make sure it is running
Make sure it is draining!
Watch it dispense AA first cycle.
Check periodically in case it runs out of AA.
Top up reagents/AA during AA coupling steps (long inactive periods).

Example of typical coupling protocol (2x, 30'):

2 ml DMF, mix 1 min, drain
2.5 ml Pip, mix 5 min, drain
3 ml Pip, mix 10 min, drain
3 ml DMF, mix 1 min, drain, repeat 3 more times
1 ml AA, 725 DIC, 725 HOBT mix 30 min, drain
3 ml DMF, mix 1 min, drain
1 ml AA, 725 DIC, 725 HOBT mix 30 min, drain
3 ml DMF, mix 1 min, drain, repeat 3 more times
1 ml Ac2O, 1 ml DIPEA, mix 5 min, drain
3 ml DMF, mix 1 min, drain, repeat 3 more times.

Post Synth Workup (Cleavage):

Resin cleavage is done with strong acid. All sidechain protecting groups will come off, and the peptide will cleave from the resin. Remaining fmoc groups will NOT come off in acid, so if you want them off, remove them with piperidine before resin-cleavage.

The NovaBiochem catalog has a lot of useful info on this subject (1999 edition, p. S52-6).

For simple cleavage, I use 88% TFA, 2% triisopropyl silane (TIS), 5% DTT, 5% H2O.

If you have either an Arg or a Trp, include some Thioanisole and Phenol (5% each?)

If you have multiple Arg, cleave for an extra long time (5-12 hrs).

It is probably a good idea to wash resin with DCM/DMF before cleavage to remove remaining piperidine salts and to swell the resin.

Transfer block to the deprotection block in the ventilated hood opposite the PepSynth machine.

Use plenty of cleavage reagent per well. I suggest 4-5 ml per well. Shake for 3 hrs to cleave (longer if multiple Arginines. Be sure to cover with amber rubber mat to prevent evaporation, especially during longer reactions. TFA is labile.)

Clean the mini-beakers that are used to collect the cleavage solution with EtOH.

Use vacuum to suck cleavage solution into mini-beakers below. Rinse resin with extra 0.5 ml TFA.

Transfer to 50 ml PolyPro conicals or 15 ml PolyPro conicals

Evaporate to small volume (0.5 ml?) using Danny's block evaporator under N2. Heat to 37C if desired.

Fill tube with Ethyl Ether (at least 15 fold, but preferably more). Peptide will ppt.

Leave tubes overnight in the spark-less freezer in Satellite lab (under the bench freezer opposite the plate washer). Peptide will ppt further.

Chill extra ether in same freezer for washes next day.

Next day: spin tubes in brushless-centrifuge (swinging bucket centrifuge in equipment room) at 4C 5-10 min at max speed (2500 rpm?). Keep on ice. Decant in hood into clean beakers or conical tubes (store in case peptide didn't ppt completely. If it wasn't well cleaved/deprotected it may not ppt). Fill tube with cold ether, vortex, spin, decant, refill, vortex, spin, decant, refill, vortex, spin, decant, airdry in hood (> 15 min). Add several ml water (5 ml?) vortex, bath-sonicate 5 min, vortex to try to dissolve it. Not all will dissolve. Freeze and lyophilize to remove final cleavage reagents and all traces of TFA.

Store crude peptide as lyophilized powder clearly marked in freezer.

Suggestions for Reverse Phase purification:

Determining solubility of peptide is tricky, especially since some of the powder in the crude mixture isn't peptide!

Think about pH! Look at peptide sequence and count the net charge at neutral pH. The crude mixture will contain peptide as a protonated TFA salt. Highly acidic peptides (net charge >= -2) will probably not dissolve without neutralization and maybe, even, slightly alkaline conditions. But be careful since high pH is bad for peptides. For basic peptides, you can try to dissolve in plain water, or water with 1% TFA or 10% Acetic Acid. For acidic peptides, add some dry sodium bicarbonate. If that isn't enough, try a little NH4OH (although watch pH and don't let it go over 10). After neutralization, often peptides can be lyophilized to powder and redissolved in plain water.

I suggest removing a small amount of dry peptide into several eppendorf tubes and performing tests. To one tube add some water and see if it dissolves. If not, add a little acid or base depending on peptide (see above). If that doesn't work, add a little acetonitrile. Try not to go over 20% ACN (check the expected HPLC elution time predicted by Peptide Companion program). If that still doesn't work, go to a new eppendorf with a little dry peptide and try some 6M guanididium HCl. If that doesn't work, I guess you'll have to use DMSO. Try 25% DMSO, then 50%, then 100%.

Filter sample through spin filter or syringe filter before injecting on column. If there is visible ppt., spin the sample down and save the pellet, then filter the sup.

Run over a shallow gradient (preferably about 5% ACN per column volume.)

Never load more than half of your crude in a single run! If you load DMSO on the column, wash a long time (15-20 min) before your gradient. If the ACN % in your sample is high, consider diluting with water before loading.

After RP purification and lyophilization, peptides are often water soluble even if they didn't seem to be before.

DR Peptide Binding Competition Assay

Tom, 1/6/00
originally by Aaron Sato, based on various other protocols from the Lit.

Binding Reaction:

Materials:

PBSTween (PBS, 0.1% BSA, PMSF, IAA, EDTA, Azide, pH near 7, 0.1% Tween 20).

(no Leupeptin or Pepstatin! AEBSF works OK instead of PMSF).

PolyPro 96 well Plates and covers (Corning 3365/3092, VWR 29444104/29442924).

Peptides and HA and Biotin-HA

Empty DR molecules!

Procedure:

Prepare a plate of peptides diluted in PBST at 2x final concentration and in 55 ul.

typical range, 10 uM to 0.6 nM HA by 8 4-fold dilutions, higher for other peps.

Leave at least one well per plate without any competitor peptide.

Prepare 2x solution of DR/BioHA (50 nM each, or less), 65 ul per well (6.5 ml per plate).

Quickly aliquot 55 ul of DR/BioHA onto the peptides series.

Incubate 37C (or RT) for 2-3 days.

Move to 4C

Assay:

Materials:

LB3.1 plate: coat Immulon-4 plate with 100 ul of 5-10 ng/ul LB3.1 in PBS(Z) 37C 1-2 hrs, wash with PBST or TBST and fill with Block (PBSZ/BSA) 37C 2 hrs or 4C o/n

Lots of TBST (TBS with 0.1% Tween)

Procedure:

Load 50 ul of Binding reaction into plates in duplicate samples. Incubate 37C 30-120 min.

Wash with TBST

Apply 100 ul/well 1:1000 Streptavidin-Europium in Delfia Assay buffer. 37C 15-60 min.

Take out Enhancement Solutiuon to warm up.

Wash with TBST

Apply 200 ul/well Room Temp Delfia Enhancement Solution. Develop for a while (see below).

During this step, a chelator in the enhancement solution removes Eu from the SA and into solution in a different chelation so that the Eu fluoresces. By Danny's report, this is stable (if protected from light and evaporation) for many hours, but it takes a while to reach equilibrium depending on mixing. If unmixed, allow at least one hour before reading. Or nutate 10 min to mix.

Additional Notes:

Don't change procedure much! Remember Tween! Don't use Leupep/Pepstat! Aaron called for triplicates, but I think duplicates are sufficient. I have seen some pretty different results from assaying at RT and 37C. L243 also works on the plate but it may see some alleles differently (jury still out). Aaron used to recommend including a Bio-HA, No DR sample on the Assay plate, but it gives zero signal and I think is unnecessary. A better control is DR with no Bio-HA. Also, You can add known amount of purified DR1-BioHA in order to quantitate the asssy, if desired.

SA-Fitc Labeling Protocol , as sent to John Altman.

Protocol:

Start with SA from Prozyme at 1 mg/ml in PBS. Add HABA from stock to a final concentration of 200 uM (2.5 fold excess over biotin binding sites). Add NaBicarb pH 9.0 (freshly made) to a final concentration 50 mM. Add FITC from a fresh DMSO stock to 170 uM (10 fold excess over protein) and add DMSO for a final concentration of 10%. Mix and incubate at 37C 1 hour. From 1M Tris pH 6.8 stock add to final concentration 50 mM to neutralize and quench. Concentrate in a Microcon 10 to 200 ul. Purify on G50 Sephadex column on benchtop (gravity flow). Collect fractions and look at OD scans 550 nm to 240 nm to identify fractions with SA-Fitc. I don't worry about the HABA from this point forward. It will be easily displaced by biotin when you add it. It does contribute slightly to the OD at 500 so you will probably want to do various controls to be able to properly quantitate the number of Fitc per SA. The above protocol yeilded 2.8 FITC/SA for me the one time that I did it. You might want to bump up the concentration of FITC to 250 uM. Note that if you use Alexa 488 succinimidyl ester you will probably need to use a LOWER concentration since SE react more readily. You may also want a lower pH.

SA-Alexa Protocol:

July 2001, Tom Cameron

Reagents:

Alexa-488 Succinimide Ester from Molecular Probes, A-20000.
    Make 30 mM in DMSO freshly before use. keep from light.
HABA, available from Sigma, H5126. We should have some in the lab.
    Make 10 mM stock in 10 mM NaOH
Streptavidin, from Prozyme, Inc.
    Solubilize in H2O to 6 mg/ml.
    Dialyze vs. 2 L 50 mM NaBicarb pH 8.5 o/n cold
    Change buffer and dialyze again o/n cold
NaBicarb solution, 50 mM pH 8.5, cold.
1 M Tris, pH 8.0
Pre-swollen Sephadex G-50 beads in PBS or PBSZ.

Protocol:

Mix reagents for final concentrations:
    SA 5 mg/ml (340 uM biotin binding sites)
    Haba 1 mM
    Alexa 488 3 mM
Store Room Temp 1.5 hours in the dark
Add 1 M Tris pH 8 to final conc 50 mM
Store 4C indefinitely in the dark.
Prepare a small (4-6 ml) gravity flow G-50 column. Wash with fresh PBS.
Load SA-Alexa sample. Collect fractions. First yellow peak should be SA-Alexa and should elute in < 1 column volume.
Check fractions with UV-Vis 540 nm to 240 nm. Pool as desired. Store 4C dark.

Data:

MW SA is 55 kDa
1 mg/ml SA is 3.2 OD 280
For Alexa-488 OD280/OD488 = 0.11
For Fitc, OD280/OD488 = 0.22
Fitc ext. cooeff: 68000 at pH 8, 58000 at pH 7
Alexa ext. coeff. 71000 at pH 8. Should be the same at pH 7.
MW Fitc is 494 g/ml
MW Alexa 488 is 643 g/mol
MW Haba 242.2 g/ml

OPA Assay for Free Amines:

Tom Cameron from Dikran Aivazian from Pierce Chemical Co. procedure, Nov. 99

Solutions:

Buy from Molecular Probes, P-2331, o-phthaldialdehyde.
OPA Stock: Make fresh OPA sln, 10 mg/ml in 95% EtOH.
Make fresh 200 mM Boric acid solution, titrate to pH 10 with KOH
(about 7 ml of 1 M KOH for final 50 ml Borate sln).
Borate/BME: Add Beta-mercaptoethanol to Borate sln for a final 0.2%.
OPA Working Sln: add 300 ul of OPA Stock to 10 ml of Borate/BME. Keep on ice.

Procedure:

-Use polypropylene plate,
-Put 100 ul of Borate/BME solution into wells
- Aliquot peptide (1-20 ul) into appropriate wells.
-In one row do two fold dilutions of known standard starting at 100 nmol of amine (this is 5 ul of 5 mM HA (which has four amines per peptide)).
-Now add 100 ul of OPA/Borate/BME to each well and pipet mix.
-Wait at least two minutes but less than 15 minutes.
-Read on Wallac Plate Reader with 355 nm/460 nm filters (Protocol OPA) within the next fifteen minutes. Slowly the signal will fade.
-Plot the Stds and read off the concentrations of your samples.

This is a pretty easy and hardy assay but not highly sensitive. Good down to about 1 nmol. You can use more or less OPA and you'll get the same results, shorter and longer times and the readings don't change much.

Double Western:

A protocol for assay of S-Tag and Hisx6 on the same membrane blot.

S-Tag is a peptide from RNase used by certain vectors from Novagen (pET-32).

Transfer to PVDF at 450 mAmps for 45 -60 min.
block with milk or bsa in PBSZ for 1/2 hr on shaker.
incubate in 10 ml TBST or PBST with 1:5000 S-Protein-AlkPhos (Novagen) *AND* 1:5000 Ni-NTA-HRP (Qiagen) for 1/2 hr or more on shaker.
Rinse and Wash 4 times with TBST.
Develop HRP reaction with Pierce SuperSignal kit

incubate membrane in 8 ml Stable Peroxide Soln + 8 ml Substrate soln. for 3 min.
remove with tweezers and place on saran wrap and fold saran wrap over.
cut-off xs saran wrap.
turn off all lights in dark room. close door. turn on red lamps and let eyes adjust.
take out small x-ray film from upper right hand drawer.
expose gel to x-ray film at approx times of 5 sec., 20 sec., 1 min, 3 min., and then 1 min. backwards (to be sure you didn't get the sides mixed up).
Be sure that on one of the longer exposures you find a way to mark the film so that you can later align the gel to the film.
Develop film 1 min in developer, wash in central reservoir, 2 min in fixer.
Allow film to dry. Keep blot wet.

Develop Alk Phos reaction with NBT/BCIP

Rinse blot a few times with TBST
incubate membrane in 10 ml Alk Phos Reaction Buffer with 70 ul NBT and 70 ul BCIP until color develops. Don't overdevelop because the color gets even darker when it dries! Rinse in large volume of water. Dry.

-tom

Acid Strip Protocol:

Stripping ligands from a cell surface without killing the cells.
Tom Cameron, Stern Lab, Oct 00.

Refs:

French et al., JBC, ’95 270(9):4334.
Haugh and Lauffenberger, J Theor Biol ’98 195(2):187.

Reagent:

Acid-strip buffer: 50 mM glycine, pH 3.0, 100 mM NaCl, 2 mg/ml polyvinylpyrollidinone. *Well chilled.*

Procedure:

Chill cells. Spin down and resuspend in ice-cold acid-strip buffer. Keep on ice for five to ten minutes, then wash twice with cold wash buffer.

Also consider a Pronase strip.
See Lucas & Germain Immunity ’96, 5:461. or H. Suzuki et al., Immunity ’95 2:413.

Related drug options: Phenyl Arsine Oxide (50 uM) will block most receptor-mediated endocytosis (mechanism not clear to me, although must involve phosphatase inhibition); Actin blockers like Cytochalasin D or Latrunculin A (better, available at Biomol); sodium azide and/or 2-deoxyglucose to run down ATP in cell.

293S cell culture

info from Phil Reeves, Khorana lab, 3-1866
I haven't actually done it except to expand and make freezes.
-Tom

Medium: DMEM/F-12 (50:50) with 10% FCS and Pen, Strep, Gln
(they will also grow, slightly less happy, in DMEM (no F-12))

store frozen like normal tissue culture cells (in medium, maybe supplemented with extra FCS, and 10% DMSO).

Thaw like normal cells into 10 ml into a T25.

Passage: remove media, replace with equal vol. Warm Ster. PBS. Remove PBS. Replace with $1/10^{th}$ vol Warm Trypsin EDTA (0.05% and 0.02% respectively). Incubate one minute. Bang cells off walls. Dilute up to original volume with Warm fresh media. Dilute 1:10 for passage.

DR typing via PCR:

Based on the method of Olerup & Zetterquist, Tissue Antigens '92:39:225.
Primers originally purchased from BSHI (BSHI Molecular special Interest group, University of Brisot, Dept. of Pathology and Microbioloy, Bristol Homoeopathic Hospital, Cotham, Bristol, BS6 6JU, Ph: 0117 9738477).
-tom

Isolate genomic DNA from samples:

I use QiaAmp Blood Kit, Cat # 29104, but there is a common Proteinase K/Phenol-Chloroform technique that should work too. Seems to work well from any kind of tissue: PBMCs, blood, cells from tissue culture, either frozen or fresh. (If frozen, wash away the DMSO). Use as many cells as you can (2-5 million seems to be great). I have even used spit. About 0.5 ml will give enough genomic DNA! Store DNA frozen at -20C

Obtain primers according to the method of Olerup & Zetterquist, Tissue Antigens '92:39:225.

Also see http://www.pam.bris.ac.uk/services/oligonuc.htm or http://www.umds.ac.uk/tissue/bshix5.html.

It is important to have some primers that act as a PCR + control. I use primers from BetaGlobin. Either pair F1 and R1 or pair F2 and R2:

BGlobinF1	GACGCAGGAAGAGATCCATCTAC
BGlobinR1	CAACTTCATCCACGTTCACC
BGlobinF2	GAAGAGCCAAGGACAGGTAC
BGlobinR2	GGTTGCCCATAACAGCATCAGG

Either pair will give a band at either 500 or 600 bp (I forget which).

I store the primers mixed together at 3X final concentration at -20 (or 4C for a short time)

Setup PCR!

Things you need:

Primers and genomic DNA.
PCR machine with a 96 well format
96 well plate for PCR
Some sort of method for sealing the PCR plate
Taq DNA polymerase
Taq buffer, dNTPs, MgCl2
A multichannel pipettor good with small volumes!
A large 2-3% agarose gel with lanes spaced for multichannel pippetor!

Each reaction should be:

40 ng of genomic DNA, about 0.25 uM forward primer (5'xx), 0.25 uM reverse primer (3'xx), 1X PCR buffer, 2 mM MgCl2, 200 uM each dNTP, and a smidgeon of Taq polymerase (0.1 units?).

Easiest way to do that for 4 genomic samples (ie a full 96 well plate):

Using multichannel, pipet 5 ul primers into wells.
Prepare a cocktail of Taq buffer, dNTPs, MgCl2, Taq polymerase:

100 ul 10X Taq buffer
100 ul dNTPs
40 ul 50 mM MgCl2
560 ul H20

3 ul Taq polymerase (5 units/ul)

Into a single column of a different 96-well plate, aliquot 98 ul of cocktail.

Select your genomic DNA samples and check their concentrations. Add 100-400 ng of the first one to each of the top two cocktail aliquots, then add H20 to make the final volume 125 ul in each. Do the same for the second genomic sample to the 3rd and 4th, and so on ...

Using multichannel, aliquot 10 ul from this genomic/cocktail mix to the columns of your plate that contains the primers. Pipet mix once, but try not to add air bubbles. Repeat for all twelve columns, with new tips each time!

Run PCR machine: 10x(96C 40 sec, 65C 40 sec) and 20x(96C 40 sec,63C 40 sec,72C 40 sec).

Run the Gel!: add 3 ul 6X Agarose loading Buffer to each sample and load your agarose gel! Run for about 30 min and look on the UV-Vis. Look for lanes which have a band around 200 bp. (Make sure all lanes have a high MW band from the control primers).

In practice, I find this harder than it looks. Some bands are weak or erratic (erratically reproduceable). But, it gives you some good leads, at least.

DR1 seems to be especially erratic. It seems to come up weakly very often, even in samples which I'm pretty sure are DR1 negative. To confirm DR1 results, I suggest an additional set of PCRs using the 5'01 primer and various 3' primers: 3'14 (all *01xx), 3'15 (not *0103), 3'10 (only *0103), 3'12 (*0101/021/04), 3'047 (*0101/04), 3'08 (*0102), 3'01 (no *01xx). And be sure to include both DR1+ and DR1- genomic samples. **Also, do a 100 ul reaction, purify by QiaQuick, and submit for sequencing with the 3' primer.**

good luck!

Tom Cameron, tomc@mit.edu, lab of Larry Stern, Aug, 2000, MIT.

Alternatives:

You can PCR all DR genes simultaneously from either total RNA or genomic DNA and use this directly for DNA sequencing or clone into plasmids and sequence the "library". You can screen e. coli colonies using the kit in order to get all the different alleles without sequencing dozens of colonies.

Primer Mixes for DR typing

rxn #	Primer name	Primer name	Alleles amplified in theory (DRB1 unless stated)	Control?
1	5'01	3'15	0101, 0102	+BGlobinF2/R2
2	5'01	3'10	0103	+BGlobinF2/R2
3	5'02	3'01	15011, 15012, 15021, 1503	+BGlobinF2/R2
4	5'02	3'02	1601-16021, 1603, 1607-08	+BGlobinF2/R2
5	5'03	3'03	03011, 03021, 0303, 0305-0308	+BGlobinF2/R2
6	5'06	3'048	03011, 03012, 1327	+BGlobinF2/R2
7	5'03	3'047	03021-022/05, 1109/20, 1302/29/31, 1402/03/09/24/27/30	+BGlobinF2/R2
8	5'04	3'047/48	0401-0407, 0410-0412, 0415, 0418, 0421, 0422, 1410	+BGlobinF2/R2
9	5'07	3'079	0701	+BGlobinF2/R2
10	5'08	3'045/18	0801-08041/05/06/10/12-14/16/17, 1317, 1411/15/28	+BGlobinF2/R2
11	5'06	3'079	09011, 09012	+BGlobinF2/R2
12	5'10	3'047	1001	+BGlobinF2/R2
13	5'05	3'06	11011-012/02-04/06/13-15/17-22/25/29	+BGlobinF2/R2
14	5'12	3'048	1201, 12031-032	+BGlobinF2/R2
15	5'03	3'10	1116, 1120, 1301, 1302, 1315, 1316, 1327, 1328, 1331	+BGlobinF2/R2
16	5'05	3'045	13031-032, 1304, 1312, 1321, 1330	+BGlobinF2/R2
17	5'03	3'17	1109, 1305, 1318, 1427	+BGlobinF2/R2
18	5'05/08	3'11	1117, 1401, 1404-05, 1411, 1414, 1423, 1426, 1428	+BGlobinF2/R2
19	5'03	3'12	1305, 1306, 1402, 1406, 1409, 1413, 1417, 1429, 1430	+BGlobinF2/R2
20	5'04	3'19	1410	+BGlobinF2/R2
21	5'51	3'01/16	DRB5*0101-02, DRB5*0201-03, DRB5*0106	+BGlobinF2/R2
22	5'52.1/.2	3'13/14	DRB3*01011/01014/0103/0201-0202/0204-06/0301	+BGlobinF2/R2
23	5'53	3'048	DRB4*01011/0102-03/0105	+BGlobinF2/R2
24	5'01	3'15	0101, 0102	+BGlobinF2/R2

A1-A12/C1-12/E1-E12/G1-G12 are rxns 1-12, B1-B12/D1-D12/F1-F12/H1-H12 are rxns 13-24

Primer mixes are 3x for PCR, 1 uM each DR primer, .5 uM Ctrl primers

Control Primers produce a 500 or 600 bp product. Some mixes don't have them!

For each reaction: 5 ul Primer Mix plus 10 ul (40 ng genomicDNA/Buffer/Taq/dNTP cocktail.

10x(96C,65C) and 20x(96C,63C,72C). Olerup Tissue Antigens 39:225. BSHI Univ of Bristol.

http://www.pam.bris.ac.uk/services/oligonuc.htm http://www.umds.ac.uk/tissue/bshix5.html

times used	Primer Name	Primer Seq
2	3'01	CCGCGCCTGCTCCAGGAT
1	3'02	AGGTGTCCACCGCGGCG
1	3'03	TGCAGTAGTTGTCCACCCG
2	3'045	TGTTCCAGTACTCGGCGCT
3	3'047	CTGCACTGTGAAGCTCTCAC
4	3'048	CTGCACTGTGAAGCTCTCCA
0	3'05	CTGCAGTAGGTGTCCACCAG
1	3'06	CTGGCTGTTCCAGTACTCCT
2	3'079	CCCGTAGTTGTGTCTGCACAC
0	3'08	CACTGTGAAGCTCTCCACAG
2	3'10	CCCGCTCGTCTTCCAGGAT
1	3'11	TCTGCAATAGGTGTCCACCT
1	3'12	TCCACCGCGGCCCGCC
1	3'13	CTGTTCCAGGACTCGGCGA
2	3'14/3'18	GCTGTTCCAGTACTCGGCAT
1	3'15	CCGCCTCTGCTCCAGGAG
1	3'16	CCGCGGCGCGCCTGTCT
1	3'17	CCCGCCTGTCTTCCAGGAA
1	3'19	CTGTTCCAGTGCTCCGCAG

times used	Primer Name	Primer Seq
2	5'01	TTGTGGCAGCTTAAGTTTGAAT
2	5'02	TCCTGTGGCAGCCTAAGAG
5	5'03	TACTTCCATAACCAGGAGGAGA
2	5'04	GTTTCTTGGAGCAGGTTAAACA
3	5'05	GTTTCTTGGAGTACTCTACGTC
2	5'06	GACGGAGCGGGTGCGGTA
1	5'07	CCTGTGGCAGGGTAAGTATA
2	5'08	AGTACTCTACGGGTGAGTGTT
1	5'10	CGGTTGCTGGAAAGACGCG
1	5'12	CATAACCAGGAGGAGCTCC
1	5'51	GTTTCTTGCAGCAGGATAAGTA
1	5'52.1	TTTCTTGGAGCTGCGTAAGTC
1	5'52.2	GTTTCTTGGAGCTGCTTAAGTC
1	5'53	GAGCGAGTGTGGAACCTGA
	BGlobinF1	GACGCAGGAAGAGATCCATCTAC
	BGlobinR1	CAACTTCATCCACGTTCACC
	BGlobinF2	GAAGAGCCAAGGACAGGTAC
	BGlobinR2	GGTTGCCCATAACAGCATCAGG

See Olerup & Zetterquist, Tissue Antigens '92:39:225-

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lots of expt detail contained in FACS data books
NB13: 10/00-10/01
lots of expt detail contained in FACS data books

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NB11: 8/99-2/00

S2 protein expn, esp DR4
Lots of FACS
Drzip folding test (don't seem to fold)
HIV pep synthe and competitions

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Compare Coli DR1 with S2 DR1 and S2 DR1 cov pep
16 FACS 8/20/99
20 Gels of Drzip Ibs crude and HQ pure
23 Rphase purify HA pep, prep scale
26 Westerns and ELISAs of S2 expn tests. Look bad
29 ELISA of Drzip expns

32 Gels of conc Drzip expns
34 Dot blot and elisas of S2 expns
40 Trying to clone S2 cells: Elisa of clone sups
Looks good, although they never pan out to be anything
46 FACS 10/4/99
48 FACS 10/14/99
50 Trying to develop an allele-nonspecific ELISA by Competition with DR1 doesn't really work
58 FACS 11/4/99
60 FACS 11/17/99
62 FACS 11/11/99 Offrate expt
68 Summary of recent work: S2 lines, expn, HIV epitope prediction and synth facs of HA03 line
70 Notes on HIV pep synth
Good Notes
74 DR Pep comp assay with HIV peps (and others)
No tween so no signal?
76 Gel of empty DR4, dR1HA coli
78 Correspondance from Lucy, DR8 cDNA
85 GF analysis of SA tets
88 DR Pep comp assay with HIV peps (and others)
90+ Various biochem work with EAF, gels, UVVis, BCA assays
105 DR Pep comp assay with HIV peps (and others)
106 Summary of recent work: S2 lines, expn, HIV epitope prediction and synth
113 HQ purify DR subunits, Setup Test Foldings
114 Elisa to get results of test foldings from p 113
116 Cloning Codes: J17-30: pRMHA3 DR52a, DR8
118 HQ purify DR subunits, Setup Test Foldings
120 Get peps from Sam poon in Walker lab. Notes
124 ELISA of test foldings from p. 118
126 AAA analysis of gag peptide to get concentration
130 Foldings of DR with HIV peps
133 DR Pep comp assay with HIV peps (and others)
136 Summary of recent work:trying to improve pep loading of DR4
140 GF analysis to see rate of SA-biotin binding is fast
144 DR Pep comp assay with HIV peps (and others)
148 More GF of SA-fitc and Drpep
150 DR Pep comp assay with HIV peps (and others)

NB12: 2/00-9/00
Making DR-HIV pep complexes
More analysis with SA, Native gels and GF
Anergy expts
SC constructs, Coli
S2 expn with DR1SS, short ProCysPro tail, diff alleles

Trying to make new short term T cell lines, clones

Page	Content
7	DR1-HIVpeps yeilds table
11	loading various DR preps with Bio-peps
	Elisa assay
16	Large set of gels DR-HIV pep complexes
	Native gels of DR-HIV pep complexes
	Native gels of ACLB1S HA with EAF
18	Some more gels and natives
22	GF of DR and SA
24	Data on SA from Prozyme. Good stuff!
30	native gel of DR and SA. Nice tight bands.
32	Gels of Drpep +/- SA.
	As a biotinylation assay. Looks pretty good but this
	isn't yet the final iteration.
35	Various complexes NB+SA
	Nice gel of the DR1HAssbio +/- bio
38	FACS around 4/12/00?
40	Trying to make DR-fluorpep complexes
45	Scans of fluor-HA/A2 peptides
48	native gel of DR and SA. Nice tight bands.
	SDSPage of DR pep complexes. Nice B/NB
50	labeling Prozyme SA with Haba and Fitc
	and a Native gel with the same. Looks pretty decent.
52	Cloning Codes X1-X28
	pRMHA with DR1 Sig Seq and various other alleles for gene.
54	More GF of DR-SA
	still looks like crap
56	Prep of DR4 from S2 cells
60	FACS 5/2/00
61	GF of DR and SA
63	FACS 5/16/00
64	FACS 5/18/00
65	FACS 5/23/00
66	FACS 5/24/00
67	fACS 5/2?/00
68	Anergy expt HA1.7 cells 5/29/00
	using OKT3 and CD28 on plate
70	Protein prep, DR52 from S2 cells
72	Another anergy expt 6/1/00
74	FACS 6/1/00
76	FACS 6/8/00
78	Tahw and stim some SL08 vials
79	titrate PHA?IL2 stocks on PBMCs
80	Looking for Anergy, Data analysis

Didn't yet have a handle on the cell-death thing
84 Try thawing/stim new SL08 vials
85 FACS 6/14/00
88 Sorting 6/16/00
The 4C samples are improperly done.
90 S2 cell transfection with Lipofectin
91 ELISA of above transfection
92 titrate PHA?IL2 stocks on PBMCs
95 Sorting 6/23/00
Thaw, Stim ne SL08 vials
102 summary of T cell cloning
all these clones died.
108 Looking for PBMCs with a detectable Prolif response to
any peptide tthat I have, HA, FluB, TT, etc.
Can't find any!
110 S2 cell transfection with Lipofectin
112 Testing the Dendritic Cells from DR1tg mouse for ability
to stim T cell clones.
Concl: they don't work.
114 Prolif of T cells to HA and 312Q and Danny's P046-08 peptide
120 Try making Vesicles with biotin-lipid and coating with SA and DR1
and using as a staining reagent.
Concl: It didn't work but I don't know if because I
didn't make proper vesicles, or vesicles don't work for staining.
127 DR typing Sue, Frank Rush, Barry Rushe, CHME
126 Summary of S2 transfections from mid July, TC 51-68
129 Summary of expn levels of S2 transfectant lines TC51-68
130 Prolif results with Danny's P046-08 peptide
131 Cloning to make the Single-Chain constructs
132 Receive Blood from 1H volunteer in Sekaly lab from Bader
Start 1HHA line
135 Prolif of T cells to HA and 312Q and Danny's P046-08 peptide
139 Expn tests of S2 lines with Short PCP tail (TC61-)142
142 Anergy expt HA1.7 Cl-1 9/27/00
stain 'em too! FACS 9/28/00
144 anergy 10/4/00, prolif and FACS 10/5/00
146 Tuning the DR1 pep comp assay with diff DR preps

NB13: 10/00-10/01
Lots of Anergy Expt.
Polyclonal line Repoertoire analysis
Trying to grow short term T cell lines, DR1 homozyg
Work with Gcohen
Some DR production
Nice gels with SA to assay biotinylation.

Page	Content
6	Anergy Expt, 10/12/00, Called 3rd Anergy
7	FACS 10/13/00
10	FACS 10/1?/00
11	Sorting 10/19/00
12	DR pep binding competition assay, big
14	FACs 10/21/00
16	Drug Data analysis
18	Pep synth, HBV, various
19	FACS 10/24/00
20	DR pep binding competition assay
25	FACS 12/9/00 with Gcohen
26	Prolif Data, titration of peptides, diff lines
	Big expt, looks good.
27	DRaBirA, NI/I, cr IB
28	FACS 12/15/00 Sorting with Gcohen
	This is the expt that we got TCR seq from!
31	FACS 1/24/01
36	gel of Drpep complexes with SA
	Nice Biotinylation Assay!!!
	Nice Gels!
39	Big expt Making Fluor SA Fitc/Alexa w and w/o Haba
41	UVVis analysis of SA-fluor fracs
44	Anergy Expt, 4/9/01 w/ Jen Stone
46	Anergy Expt 4/16/01
48	Anergy Expt datat analysis
50	DR-pep production notes/summary
51	Gels of DR-pep reagents, +/- SA. Nice.
	Nice Gels!
52	GF of SA with DR.
54	Trying to stimulate DR1 Homozygous cells that
	I got from George Cohen (volunteer Scott Bercury)
58	Anergy Expt 4/30/01
60	Stimulate cells from Scott Bercury, new batch
	trying to get these cells for work with Yuri, but never worked out
66	Anergy expt. 5/14/01
70	FACS 6/11/01
71	FACS 6/11/01
73	Anergy 6/18/01
74	GF on Danny's Water's HPLC with WISP!!!!
	Summary of injections
	Overlay plots, titrations.
	Nice expt!!!
76	Test the Folding Beads from Alan Fersht for IgBeta/IgAlpha. Didn't help!

Spiral-Bound FACS data Notebook Tables of Content

Tom Cameron, Stern Lab, 8/01

All my FACS data except for the very very earliest is in these binders

Five 3-ring binders:

FACS-1: Flu Study, 12/98-2/99
Lots of experiments trying to stain blood samples from Stern Lab or Aviron Flu Study
All amounted to Nothing in the end!!!

FACS-2: 4/99-8/00 FACS DATA

FACS-3: 9/00-12/00, More FACS data

FACS-4: 12/00-2/01, And 8/01, FACS 2001 TomC

FACS-5: 4/01-8/01, 2001 Anergy Expts

FACS-2: 4/99-8/00 FACS DATA

Stapled packet of data for Aug 19, 98: Early simple expt with HA1.7, Cl-1, CH7C17, Jurkats
to see staining, with various controls.
4/15/99: SL volunteers pre and post boost
Can we get FRET in the FACS? Not in this expt
Azide and TCR downreg: no apparent effect.
4/26/99: Acidic compartment? Fitc vs Bodipy
Acid Strip
Drug inhibition of Staining
4/22/99: Staining cells from boosted SL volunteers after short term culture
may have been bad 307R-bio reagent
4/29/99: Staining cells from boosted SL volunteers after short term culture
may have been bad 307R-bio reagent
4/29/99B: Titrate HA1.7, Cl-1 for Tet Stain, CD3-downreg
Looks Good
5/6/99: Stain cells from boosted SL volunteers after short term culture
Realize 307R reagent is bad.
5/9/99: Notes to summarize current progress
xeroxes of experimental charts for data workup
5/12/99: HA1.7 Fluorescein vs. Bodipy Staining effects
5/13/99: HA1.7, PAO titration

Stain SL polyclonals with Tet and think I see some signal
but cells are nearly all dead.
5/20/99: Stain SL polyclonals and seem to see some signal, bigger
5/27/99: Stain SL polyclonals, day 0 and day 13 samples
see staining in SL08, day 13 307R stim samples
Memory phenotype (45RO+/62L-)
Drug effects on HA1.7 staining; PAO, CytoD, MBCD
Neutralization Expt: SA-Fitc,OrGr,Bodipy, +/- NH4Cl
6/10/99: Neutralization with Chloroquine, Monensin, NH4Cl
CytoD, Lat A, PP2, Genestein, Quercitin ttrns
6/24/99: Neutralization, Ttrn
6/25/99: Neutralization, Ttrn, Bad Expt, cells are dead.
6/30/99: Fitc-Dextran uptake effect of Tet staining? None detected.
staining S2 DR transfectants: No good
7/2/99: staining S2 DR transfectants: No good.
7/9/99: staining S2 DR transfectants and sorting: no good
7/12/99: Time course of HA1.7 staining. Looks pretty good.
7/22/99: Stianing SL08-7-R line (mostly dead)
Staining HA1.7 and Cl-1 at 37C and 4C, various controls
7/23/99: Jovi-Fab internalization/Tet Staining experiment.
Look at Again.
7/26/99: Temp titration HA1.7, Cl-1
8/14/99: Try different ways of gating out monocytes, CD14/64/8/19
8/12/99: More monocyte-gating,
stain HA1.7 and Cl-1 with HA312Q: no staining!
8/13/99: S2 DR transfectants sorting: No good.
9/15/99: HA1.7 with Drugs (incl TubC/T) and Short Term line with drugs.
10/4/99: HA1.7 and HA03 with drugs
10/27/99: Try Off-rate Expt! HA1.7, HA03
Also, try SA magnetic beads for staining
11/4/99: More offrate data
Drugs and HA1.7, HA03
DATA SUMMARY Drugs and Off rate
11/11/99: HA1.7/Cl-1 Offrate expt
11/18/99: More offrate, Thapsigargin
4/24/00: trying to strip with DTT/Bmesa
4/25/00: Stripping with Bmesa
5/2/00: Stripping with Bmesa
5/3/00: Stripping with Bmesa, Tet vs Ab
5/9/00: Data with Lucy in London
5/11/00: Data with Lucy in London
DATA SUMMARY of work with Lucy
5/16/00: Stripping data tet vs ab, Striping Fitc-Tets
5/18/00: Stripping data tet vs ab, Cl-1
5/23/00: Stripping data tet vs ab
5/24/00: Overnight staining of Cells

Stain with PE tet, follow with APC tet expt
Stripping Tet vs Ab
5/25/00: 2-deoxyglu inhibition
Stripping FitcTets
6/1/00: 2-deoxyglu+Azide, PAO preinc, 4C, SA-Fitc PAO/4C, wash free SA from SAPE
6/12/00: SL08 prolif, CFSE staining
6/14/00: Staining PBMCs, titrate CFSE, PHA, IL2
6/21/00: Staining newly thawed HA03, CH7C17
6/16/00: Sorting: after CFSE labelling and stimulating SL08 PBMCs
6/23/00: Staining/Sorting
SL08 cells CFSE labelled and stimulated, stained and Sorted
No signal.
7/4/00: RBLs with HA1.7-zeta (Y22.D6)
7/5/00: 37C v 4C staining, HA1.7 Cl-1 HA03, Y22.D6
8/7/00: Detailed analysis of HA03 line: 37v4, Phenotype
8/9/00: More analysis of HA03 line, Phenotype, etc.

FACS-3: 9/00-12/00, More FACS data

9/14/00: 37C v 4C, HA1.7, Cl-1, H2, H8
9/21/00: testing FRET in FACS and NoWash staining bk
9/20/00: Try PreStim/PreAgg effects on staining
titrate density of cells in well effect on staining
9/22/00: Staining Stim. 1HHA line
9/25/00: Drug data, HA1.7 cells
10/2/00: Anergy Expt, HA1.7, Cl-1 First
10/5/00: Anergy Expt HA1.7, Cl-1, HA03 Second
10/8/00: Stain 1HHA polyclonal after two stims, 37C v 4C
10/16/00: Anergy Expt, HA1.7, Cl-1, HA03, Third
10/16/00: Drug Expt HA1.7, Cl-1, various plus Fitc-Dextran.
10/19/00: Drug Expt HA1.7, plus Fitc-Dextran, CarboxyFluor, MBCD+/-Chol
10/21/00: Drug Expt, Large, HA1.7, Cl-1, HA03, Cfluor,
Also, CD4-blocking antibodies
10/26/00: Drug Expt, HA1.7, HA03, Cfluor
DRUG DATA SUMMARY
10/24/00: 1HHA, 1HFB, SL07, SL08 staining after two/one stimulations
10/24/00: SL07, SL08, 1HHA freshly thawed PBMCs
11/1/00: Drugs expt, HA1.7, confirm Act. D result
11/7/00: Various Polyclonal Stainings, 37C, 4C, Lat A, including first FluBTet staining
11/10/00: Various Polyclonal stainings and Prolifs
with pre-agg using anti-TCR
11/16/00: Titrating new CFSE batch
11/22/00: Various polyclonals, 37C v 4C
and staining the PHA stim vs Pep stim lines

11/26/00: Titrate oligomer on various polyclonals with parallel prolif. Messy Expt.
11/27/00: Titration of CFSE
11/28/00: Expt with George Cohen: Tet analysis at Day 0. High BK!
11/29/00: Expt with George Cohen, Ifng analysis
12/1/00: Various cells, titrate new batch of SAPE (lot 1401), diff DR1HA batches
12/6/00: Staining Titration various lines: HA1.7, Cl-1, HA03, 1HTet+/-, 1HHA, H8
37C v 4C
12/9/00: Expt with George Cohen, staining stim samples on day 11, Look Good!

FACS-4: 12/00-2/01, And 8/01, FACS 2001 TomC

12/16/00: Expt with George Cohen, Ifn-gamma staining
1/11/01: Data from Philip Norris, staining of HIV line
1/24/01: Drug Expt on 1HHA Tet+ and 1HFB
2/8/01: Sorting, DFCI, 1HHA2 cells line, 37C, 4C
1/31/01: Data from George Cohen, TCRVbeta mAb analysis data
12-2/01: Data from Philip Norris, Staining with HIV tets
2/01: TCR sequences from George Cohen
8/8/01: Titrate tet, 37C, 4C, 1HHA, 1HFB, HA03, CH7C17
And do TCR Vbeta analysis of 37C and 4C Tet+ populations
8/9/01: Anergization for 1 week, then titration

FACS-5: 4/01-8/01, 2001 Anergy Expts

4/9/01: Prolif assay to determine optimal anergization conditions, HA1.7, 1HHA2
4/16/01: Anergization of 1HHA2, Staining Time Course
5/1/01: Anergize HA1.7, H8, Cl-1, Prolf and Staining
5/17/01: IL-2 Intracellular Cytokine Assay Attempt
6/1/01: IL-2 Intracellular Assay
Anti-Fas antibody protection from apoptosis?
HA03 anergization and Staining
6/6/01: IL-2 assay
Anergizing CH7C17 cells
6/14/01: Staining and Prolif of Various Cell Lines: HA1.7, Cl-1, H8, HA03, 1HHA, 1HFB
37C and 4C although I used the over-biotinylated DR1HA so 4C data is high
6/12/01: Trying to Anergize CH7C17 cells
6/19/01: Anergizing HA03 cells, various stimuli, very weak prolif signal
6/22/01: Anergize HA03, 1HHA, 1HFB, CH7C17, Stain, Prolif
Scatter plots CD3 v Tet
7/5/01: Anergize 1HHA, CH7C17, then Prolif and Titrate Tetramer for Kx
7/10/01: Anergize 1HHA, then Prolif and titrate Tetramer for Kx
8/4/01: Stain various lines and analyze Vbeta rep with mAb

pLMI AS/A1S

for DRAlphaShort: truncated, extracellular domain only, no connecting peptide.

gaattcaggaggaatttaaa**ATG**ATCAAAGAAGAACATGTGATCATCCAGGCCGAGT
TCTATCTGAATCCTGACCAATCAGGCGAGTTTATGTTTGACTTTGATGGTGA
TGAGATTTTCCATGTGGATATGGCAAAGAAGGAGACGGTCTGGCGGCTTGA
AGAATTTGGACGATTTGCCAGCTTTGAGGCTCAAGGTGCATTGGCCAACAT
AGCTGTGGACAAAGCCAACTTGGAAATCATGACAAAGCGCTCCAACTATACT
CCGATCACCAATGTACCTCCAGAGGTAACTGTGCTCACGAACAGCCCTGTG
GAACTGAGAGAGCCCAACGTCCTCATCTGTTTCATCGACAAGTTCACCCCA
CCAGTGGTCAATGTCACGTGGCTTCGAAATGGAAAACCTGTCACCACAGGA
GTGTCAGAGACAGTCTTCCTGCCCAGGGAAGACCACCTTTTCCGCAAGTTC
CACTATCTCCCCTTCCTGCCCTCAACTGAGGACGTTTACGACTGCAGGGTG
GAGCACTGGGGCTTGGATGAGCCTCTTCTCAAGCACTGGGAGTTTGATGCTt
aaaagctt

MIKEEHVIIQAEFYLNPDQSGEFMFDFDGDEIFHVDMAKKETVWRLEEFGRFAS
FEAQGALANIAVDKANLEIMTKRSNYTPITNVPPEVTVLTNSPVELREPNVLICFI
DKFTPPVVNVTWLRNGKPVTTGVSETVFLPREDHLFRKFHYLPFLPSTEDVYD
CRVEHWGLDEPLLKHWEFDA*

pLMI ACL

for DRAlphaCysLong: Extracellular DRalpha with connecting peptide & C-Ter Cysteine.

gaattcaggaggaatttaaa**ATG**ATCAAAGAAGAACATGTGATCATCCAGGCCGAGT
TCTATCTGAATCCTGACCAATCAGGCGAGTTTATGTTTGACTTTGATGGTGA
TGAGATTTTCCATGTGGATATGGCAAAGAAGGAGACGGTCTGGCGGCTTGA
AGAATTTGGACGATTTGCCAGCTTTGAGGCTCAAGGTGCATTGGCCAACAT
AGCTGTGGACAAAGCCAACTTGGAAATCATGACAAAGCGCTCCAACTATACT
CCGATCACCAATGTACCTCCAGAGGTAACTGTGCTCACGAACAGCCCTGTG
GAACTGAGAGAGCCCAACGTCCTCATCTGTTTCATCGACAAGTTCACCCCA
CCAGTGGTCAATGTCACGTGGCTTCGAAATGGAAAACCTGTCACCACAGGA
GTGTCAGAGACAGTCTTCCTGCCCAGGGAAGACCACCTTTTCCGCAAGTTC
CACTATCTCCCCTTCCTGCCCTCAACTGAGGACGTTTACGACTGCAGGGTG
GAGCACTGGGGCTTGGATGAGCCTCTTCTCAAGCACTGGGAGTTTGATGCT
CCAAGCCCTCTCCCAGAGACTACAGAGAACcaacctgcatgctaaaagctt

MIKEEHVIIQAEFYLNPDQSGEFMFDFDGDEIFHVDMAKKETVWRLEEFGRFAS
FEAQGALANIAVDKANLEIMTKRSNYTPITNVPPEVTVLTNSPVELREPNVLICFI
DKFTPPVVNVTWLRNGKPVTTGVSETVFLPREDHLFRKFHYLPFLPSTEDVYD
CRVEHWGLDEPLLKHWEFDAPSPLPETTENQPAC*

pLMI DRABirA

for DRAlpha with BirA biotinylation site: Extracellular DRalpha with connecting peptide and C-Terminal BirA site.

ctagaaataattttgttgaattgctagcaggaggaatttaaa**ATG**ATCAAAGAAGAACATGTGATC
ATCCAGGCCGAGTTCTATCTGAATCCTGACCAATCAGGCGAGTTTATGTTTG
ACTTTGATGGTGATGAGATTTTCCATGTGGATATGGCAAAGAAGGAGACGG
TCTGGCGGCTTGAAGAATTTGGACGATTTGCCAGCTTTGAGGCTCAAGGTG
CATTGGCCAACATAGCTGTGGACAAAGCCAACTTGGAAATCATGACAAAGC
GCTCCAACTATACTCCGATCACCAATGTACCTCCAGAGGTAACTGTGCTCAC
GAACAGCCCTGTGGAACTGAGAGAGCCCAACGTCCTCATCTGTTTCATCGA
CAAGTTCACCCCACCAGTGGTCAATGTCACGTGGCTTCGAAATGGAAAACC
TGTCACCACAGGAGTGTCAGAGACAGTCTTCCTGCCCAGGGAAGACCACCT
TTTCCGCAAGTTCCACTATCTCCCCTTCCTGCCCTCAACTGAGGACGTTTAC
GACTGCAGGGTGGAGCACTGGGGCTTGGATGAGCCTCTTCTCAAGCACTG
GGAGTTTGATGCTCCAAGCCCTCTCCCAGAGACTACAGAGGGATCCCTGCA
TCATATTCTGGATGCACAGAAAATGGTGTGGAATCATCGATAAAGATCTAAG
CTT

MIKEEHVIIQAEFYLNPDQSGEFMFDFDGDEIFHVDMAKKETVWRLEEFGRFAS
FEAQGALANIAVDKANLEIMTKRSNYTPITNVPPEVTVLTNSPVELREPNVLICFI
DKFTPPVVNVTWLRNGKPVTTGVSETVFLPREDHLFRKFHYLPFLPSTEDVYD
CRVEHWGLDEPLLKHWEFDAPSPLPETTEGSLHHILDAQKMVWNHR*

pLMI B1S

for DRB1*0101Short: truncated, extracellular domain only, no connecting peptide.

gaattcaggaggaatttaaa**ATG**GGGGACACCCGACCACGTTTCTTGTGGCAGCTTA
AGTTTGAATGTCATTTCTTCAATGGGACGGAGCGGGTGCGGTTGCTGGAAA
GATGCATCTATAACCAAGAGGAGTCCGTGCGCTTCGACAGCGACGTGGGG
GAGTACCGGGCGGTGACGGAGCTGGGGCGGCCTGATGCCGAGTACTGGA
ACAGCCAGAAGGACCTCCTGGAGCAGAGGCGGGCCGCGGTGGACACCTA
CTGCAGACACAACTACGGGGTTGGTGAGAGCTTCACAGTGCAGCGGCGAG
TTGAGCCTAAGGTGACTGTGTATCCTTCAAAGACCCAGCCCCTGCAGCACC
ACAACCTCCTGGTCTGCTCTGTGAGTGGTTTCTATCCAGGCAGCATTGAAGT
CAGGTGGTTCCGGAACGGCCAGGAAGAGAAGGCTGGGGTGGTGTCCACAG
GCCTGATCCAGAATGGAGATTGGACCTTCCAGACCCTGGTGATGCTGGAAA
CAGTTCCTCGGAGTGGAGAGGTTTACACCTGCCAAGTGGAGCACCCAAGTG
TGACGAGCCCTCTCACAGTGGAATGGAGAGCATAAaagctt

MGDTRPRFLWQLKFECHFFNGTERVRLLERCIYNQEESVRFDSDVGEYRAVTE
LGRPDAEYWNSQKDLLEQRRAAVDTYCRHNYGVGESFTVQRRVEPKVTVYPS
KTQPLQHHNLLVCSVSGFYPGSIEVRWFRNGQEEKAGVVSTGLIQNGDWTFQ
TLVMLETVPRSGEVYTCQVEHPSVTSPLTVEWRA*

pLMI BCL

for DRB1*0101CysLong: truncated, extracellular domain only, connecting peptide, AlaCys.

gaattcaggaggaatttaaa**ATG**GGGGACACCCGACCACGTTTCTTGTGGCAGCTTA
AGTTTGAATGTCATTTCTTCAATGGGACGGAGCGGGTGCGGTTGCTGGAAA
GATGCATCTATAACCAAGAGGAGTCCGTGCGCTTCGACAGCGACGTGGGG
GAGTACCGGGCGGTGACGGAGCTGGGGCGGCCTGATGCCGAGTACTGGA
ACAGCCAGAAGGACCTCCTGGAGCAGAGGCGGGCCGCGGTGGACACCTA
CTGCAGACACAACTACGGGGTTGGTGAGAGCTTCACAGTGCAGCGGCGAG
TTGAGCCTAAGGTGACTGTGTATCCTTCAAAGACCCAGCCCCTGCAGCACC
ACAACCTCCTGGTCTGCTCTGTGAGTGGTTTCTATCCAGGCAGCATTGAAGT
CAGGTGGTTCCGGAACGGCCAGGAAGAGAAGGCTGGGGTGGTGTCCACAG
GCCTGATCCAGAATGGAGATTGGACCTTCCAGACCCTGGTGATGCTGGAAA
CAGTTCCTCGGAGTGGAGAGGTTTACACCTGCCAAGTGGAGCACCCAAGTG
TGACGAGCCCTCTCACAGTGGAATGGAGAGCACGGTCTGAATCTGCACAGA
GCAAGGCATGCTAAAAGCTT

MGDTRPRFLWQLKFECHFFNGTERVRLLERCIYNQEESVRFDSDVGEYRAVTE
LGRPDAEYWNSQKDLLEQRRAAVDTYCRHNYGVGESFTVQRRVEPKVTVYPS
KTQPLQHHNLLVCSVSGFYPGSIEVRWFRNGQEEKAGVVSTGLIQNGDWTFQ
TLVMLETVPRSGEVYTCQVEHPSVTSPLTVEWRARSESAQSKAC*

pLMI vector

used for T7 expression of our constructs. Our constructs are cloned between EcoRI and HindIII. Induce in BL21 DE3 (+/- pLys, 0.2% D-Glucose) just like any pET vector with 0.5 mM IPTG when cells are about 0.7-1.5 OD.

ACTTTGTCTTCCCCCTTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGAC
ACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGG
AGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGG
GCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATA
TGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGC
GAAATTGTAAACGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATC
AGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAA
AGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCC
ACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCA
GGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTC
GAGGTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAG
AGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAA
GCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGC
GCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCG
CGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCG
GGCCTCTTCGCTATTACGCCAGCTGCGAAATTAATACGACTCACTATAGGGA
GACCACAACGGTTTCCCTCTAGAAATAATTTTGTT**GAATTC**GAGCTCGGTAC
CCGGGGATCCTCTAGAGTCGACCTGCAGGCATGC**AAGCTT**GGCTGTTTTGG
CGGATGAGAGAAGATTTTCAGCCTGATACAGATTAAATCAGAACGCAGAAG
CGGTCTGATAAAACAGAATTTGCCTGGCGGCAGTAGCGCGGTGGTCCCAC
CTGACCCCATGCCGAACTCAGAAGTGAAACGCCGTAGCGCCGATGGTAGT
GTGGGGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAAC
GAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGT
GAACGCTCTCCTGAGTAGGACAAATCCGCCGGGAGCGGATTTGAACGTTGC
GAAGCAACGGCCCGGAGGGTGGCGGGCAGGACGCCCGCCATAAACTGCC
AGGCATCAAATTAAGCAGAAGGCCATCCTGACGGATGGCCTTTTTGCGTTT
CTACAAACTCTTTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCAT
GAGACAATAACCCTGATAAATGCTTCAATAATCTGCATTAATGAATCGGCCA
ACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGC
TCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCT
CACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGA
AAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGC
CGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAA
AAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATA
CCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCT
GCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCT
TTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCC
AAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTT
ATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCC
ACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCG
GTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGA

CAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGT
TGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTT
GTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCT
TTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAG
GGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAAT
TAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGA
CAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTT
CGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGG
GAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACG
CTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGA
GCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGT
TGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTT
GTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCT
TCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGT
TGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTA
AGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCT
TACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACC
AAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCG
TCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCA
TTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGA
GATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTT
TACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGC
AAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTT
TTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACAT
ATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCC
CGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCT
ATAAAAATAGGCGTATCACGAGGCCCTTTCGTCCCCT

Insect cell DR constructs:

DRalpha core:
ATGGCCATAAGTGGAGTCCCTGTGCTAGGATTTTTCATCATAGCTGTGCTGATGAGCGCTCA
GGAATCATGGGCTATCAAAGAAGAACATGTGATCATCCAGGCCGAGTTCTATCTGAATCCTG
ACCAATCAGGCGAGTTTATGTTTGACTTTGATGGTGATGAGATTTTCCATGTGGATATGGCAA
AGAAGGAGACGGTCTGGCGGCTTGAAGAATTTGGACGATTTGCCAGCTTTGAGGCTCAAGG
TGCATTGGCCAACATAGCTGTGGACAAAGCCAACTTGGAAATCATGACAAAGCGCTCCAACT
ATACTCCGATCACCAATGTACCTCCAGAGGTAACTGTGCTCACGAACAGCCCTGTGGAACTG
AGAGAGCCCAACGTCCTCATCTGTTTCATCGACAAGTTCACCCCACCAGTGGTCAATGTCAC
GTGGCTTCGAAATGGAAAACCTGTCACCACAGGAGTGTCAGAGACAGTCTTCCTGCCCAGG
GAAGACCACCTTTTCCGCAAGTTCCACTATCTCCCCTTCCTGCCCTCAACTGAGGACGTTTA
CGACTGCAGGGTGGAGCACTGGGGCTTGGATGAGCCTCTTCTCAAGCAtTGGGAaTTcGATG
CTCCAAGCCCTCTCCCAGAGACTACAGAGAACtaa

DRbeta core:
ATGGTGTGTCTGAAGCTCCCTGGAGGCTCCTGCATGACAGCGCTGACAGTGACACTGATGG
TGCTGAGCTCCCCACTGGCTTTGGCTGGGGACACCCGACCACGTTTCTTGTGGCAGCTTAA
GTTTGAATGTCATTTCTTCAATGGGACGGAGCGGGTGCGGTTGCTGGAAAGATGCATCTATA
ACCAAGAGGAGTCCGTGCGCTTCGACAGCGACGTGGGGGAGTACCGGGCGGTGACGGAG
CTGGGGCGGCCTGATGCCGAGTACTGGAACAGCCAGAAGGACCTCCTGGAGCAGAGGCGG
GCCGCGGTGGACACCTACTGCAGACACAACTACGGGGTTGGTGAGAGCTTCACAGTGCAG
CGGCGAGTTGAGCCTAAGGTGACTGTGTATCCTTCAAAGACCCAGCCCCTGCAGCACCACA
ACCTCCTGGTCTGCTCTGTGAGTGGTTTCTATCCAGGCAGCATTGAAGTCAGGTGGTTCCG
GAACGGCCAGGAAGAGAAGGCTGGGGTGGTGTCCACAGGCCTGATCCAGAATGGAGATTG
GACCTTCCAGACCCTGGTGATGCTGGAAACAGTTCCTCGGAGTGGAGAGGTTTACACCTGC
CAAGTGGAGCACCCAAGTGTGACGAGCCCTCTCACAGTGGAATGGAGAGCACGGTCTGAAT
CTGCACAGAGCAAGtaa

Note that these are the same as the core of the E. coli constructs with one exception: there is an EcoRI site in DRalpha. There might be a few other silent mutations, but they don't seem to make any difference, codon frequencies are still fine, so I've lost track exactly.

pRMHA-3:

vector for expressing in S2 cells from Nucleic Acids Research, Bunch et al., 1988 16:3, p. 1043. I don't have an electronic version of this plasmid sequence. Vector is 3849 bases

Metallothionine promotor starts at position 2:
GTTGCAGGACAGGATGTGGTGCCCGATGTGACTAGCTCTTTGCTGCA...

Polclonal site reads
GAATCATCTCAGTGCAACTAAAGGGGGGAATTCGAGCTCGGTACCCGGGG
ATCCTCTAGAGTCGACCTGCAGGCATGCAATTCGATGCACACTCACATTCTT
CTCCTAATACGATAATAAAACTTTCCATGAAAAATATGGAAAAATAT
Which includes EcoRI, SacI, KpnI, AvaI, SmaI, Xma I, BamHI, SalI unique sites.
And an XbaI which is 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.

Vector carries an Amp-R gene for passage in E. coli. No Res. Gene for S2 cells.

Baculovirus double vectors:
pVL1393 vector with a destroyed Bam/Bgl splicing site, MfeI, NcoI at the ATG, then Alpha gene, Stop, EcoRV, more pVL1393, then BamHI, Beta gene, Stop, Xba, KpnI, more pVL1393.

BaculoPlasmids: A104 (ACLB1S), A202 (ACLB1S), A107 (ACLBCL), A178 (ASBCL).

S2 vectors:
pRMHA-3 are ccloned EcoRI upstream and BamHI downstream.

Alpha with Cys ends
GAATTCGATGCTCCAAGCCCTCTCCCAGAGACTACAGAGAACCAACCTGCAT
GCTAA

Beta with cys ends
ACAGTGGAATGGAGAGCACGGTCTGAATCTGCACAGAGCAAGGCATGCTAA

From Betsy Mellins (originally Dennis Zaller) we've got DRalpha, DRbeta0101
Plasmid #196: is pRMHA-3 with DRalpha cloned symmetrically into BamHI reading
GGATCCTATAAAT**ATG**GCCATAAGTGGAGTCCCTGTGCTAGGATTTTTCATCA
TAGCTGTGCTGATGAGCGCTCAGGAATCATGGGCTATCAAAGAAGAACATGT
GATCA.....CAAAGCCAACCTGGAAATCATGACA.....TCTTCTCAAGCACTGGGA
GTTTGATGCTCCAAGCCCTCTCCCAGAGACTACAGAGAAC**TAA**GGATCC

Plamsid #197: is pRMHA-3 with DRbeta cloned symmetrically into BamHI reading
GGATCCTATAAA**ATG**GTGTGTCTGAAGCTCCCTGGAGGCTCCTGCATGACAGC
GCTGACAGTGACACTGATGGTGCTGAGCTCCCCACTGGCTTTGGCTGG.....
CAAGTGTGACGAGCCCTCTCACAGTGGAATGGAGAGCACGGTCTGAATCTGC
ACAGAGCAAG**TAA**GGATCC

Cloning S2 vectors double constructs and Ecoli DR genes with zippers, 3/99.

See pages NB10, pp10-15, 26-31, 34-6.

Have: pRMHA3, #196 (pRMHA3 DRA), #260 (pRMHA3 DRA-flag), #262 (pRMHA3 DRB1 kt3), #197 (pRMHA3 DRB1), #285 (pRMHA3 B1*0301 kt3), #276 (pRMHA3 *0402 kt3), #282 (pRMHA3 *0404 kt3).

Want: pRMHA3 DR-ACL
pRMHA3 DRB1*0401, 1501, B5*0101, and alleles from SL01, 03, 06, 10, 14
double pRMHA3 vectors containing DR-ACL and various DRB1 genes. (0101, 0301, 0401, 0402, 0404, 1501, B5*0101)
pRMHA3 DRA-AZC, DRA-AZ, DRA-BZ.
pLMI DRA-AZC (AcidZipCys), DRA-AZ, DRA-BZ, DRB-AZ, DRBBZ.

Get (none of these have been sequenced or even carefully restriction mapped!):

These genes will have pRMHA3 MCS (Eco, Sac, Kpn) up to Bam, then NcoI thru ATG, and KpnI and SalI after stops.

E141-2: pRMHA3 plus B1*0401 via Bam Sal PCRed with BacFlyBFor and GenDRBRev from pBMN 0401 cDNA from Namrata Patil

E143-4: same with B5*0101 from cDNA from Rafik's lab (JP Fortin, I think?)

E145: same with B1*1501 from cDNA from Rafik's lab.

E226-7: pRMHA3 cut EcoRI/HincII and ligated with A202 MfeI/EcoRV to produce ACL in pRMHA3. So gene has a NcoI thru ATG, and a NotI downstream of Stop codon.

The next three clones were mis-done because there is a SacI site in the SS of DRB. So all these genes are of the form Promoter-SS-DRA-Promoter-truncSS-DRB-PolyA. They are made by PCR of pRMHA3 promoter with pRMHA3for/BacFlyBrev and PCR of DRACL with BacFlyAFor/FlyACLrev, and then overlap extension and cloning via MfeI/SacI into parent vectors.

E207, 210: E142 (B1*0401) cut Eco/SacI and ligated with DRACL/Promoter MfeI/SacI. Produces Truncated DRB gene (Taq). E210 is probably not correct, by Restriction.

E217: E144 (B5*0101) cut Eco/SacI and ligated with DRACL/Promoter MfeI/SacI. Produces Truncated DRB gene (Taq).

The next series of clones have not even been restriction mapped. I cut HindIII from E207 or E217 to insert into HindIII CIP of parent vector. It seems that kt3 tag (#285,#276,#282) must have a HindIII site.

These doubles should be pRMHA3Prom-Eco/Mfe(destroyed)-NcoI-ATG-SS-ACL-Not-Pst(notunique)-PvuII-pRMHA3Prom-Eco-MCS-Bam-NcoI(not for mellins' constructs)-ATG-SS-DRB-(Bam in some cases)-SalI-PolyA and there are HindIII and EagI sites in each Promotor region.

E302,4: #197 (pRMHA3 B1*0101 cloned) with ACL from E207 **E305,7,8:** same from E217

E333,5,6: E141 (0401) with ACL from E207 **E337-40:** same from E217

E341-4: E142 (0401) with ACL from E207 **E346-8:** same from E217

E349-52: E143 (B5*0101) with ACL from E207 **E353-6:** same from E217

E357,8,60: E144 (B5*0101) with ACL from E207 **E361-4:** same from E217

E365-8: E145 (B1*1501) with ACL from E207 **E370,2:** same from E217

E373-4: pRMHA3 with ACL from E207 **E376:** pRMHA3 with ACL from E217

The next series were made from RNA from frozen PBMC of SL01, 3, 6, 10, 14 volunteers via Gibco kit and PCRed using BacFlyBfor, GenDRBrev primers and Taq polymerase. Cut Bam Sal into pRMHA3. Final vectors should be pRMHA3 vector with entire MCS (including Eco, Sac, Kpn sites) and gene inserted between Bam and Sal. Gene has NcoI thru ATG and ends with KpnI, SalI. By PCR screen, at least 393, 395, 396, 403-6, 410-2, 418-9 have good inserts.

E377-81: SL01 **E382-7:** SL03 **E388-9:** SL06 **E390-2:** SL14 **E393-402:** SL01
E403-8: SL03 **E409-16:** SL06 **E417-24:** SL10 **E425-32:** SL14

SL01	*0701, *1601/1602, B4*0101, B5*0101-0202		
SL03	*11011/1102/11041	*1301/02/06	B3*0101-0301
SL06	*1305		B3*0101-0301
SL10	*1301/02/06 *0701	B4*0101	B3*0101-0301
SL14	*11011/1102/11041		B3*0101-0301

E433-4: E226 (pRMHA3 ACL) cut PmlI PvuII CIP and ligated with PmlI PvuII from E231 (basiczip). Could be backwards. These genes will now have a HindIII site just downstream of Stop codons.

E435-6: E226 with piece from E232. Could be backwards.

E437-8: E227 with piece from E231. Could be backwards. **E439-40:** E227 wit piece from E232. Could be backwards.

E147: N131 (IgAlpha-Acid) cut Eco/Hind and replaced with PCR of DRA-AcidCys (DRABspEIAcidrev, BspEIAcidCysfor, AcidLinkCysStpRev primers) using Taq

E149: same but with Beta (using DRBBspEIAcidRev) to make DRB-AZC.

E151-2: N131 cut Eco/BspEI and ligated with PCR of DRA (DRABspEIAcidRev). To make DRA-AZ

E231-2: N144 (Igbeta-basiczip) cut Xba/BspEI and ligated with E147 XB to produce pLMI DRA-BZ. Hopefully.

E235: N144 cut Xba/SacI and ligated with PCR of DRB cut Xba/SacI (using DRBBspEIAcidRev primer) to make DRB-BZ.

Need: Sort out clones from SL01,03,06,10,14. Create double vectors with these. pRMHA3 DRA-AZ, DRA-AZC.

Send for DNA sequencing: E141 with BetaSeqFor, BetaSeqRev, E143 with 5'51, BetaSeqRev, E145 with 5'02, BetaSeqRev, E207, AlphaSeqRev, AlphaSeqFor, BetaSeqRev, E147 AlphaSeqFor, AlphaSeqRev, E149, BetaSeqFor, BetaSeqRev, and one of E433-6 with AlphaSeqFor.

Cloning, May 2000, Tom and Deepali

Used for the eventual **plasmids X1 to X18**
See Tom's Notebook #12, pages 49-53, and Deepali's Notebook: #22, pages 46 to 50.

Goals:

pRMHA3 with extracellular DRB1*0801 gene
pRMHA3 with various extracellular DRB genes and DR1SS
pRMHA3 with DRAlpha, short connecting peptide, and ProCysPro
(Call this pRMHA3 ACS).

PCR:

LongBFor is a very long primer that has BamHI, the entire DR1 SS, and anneals to the expressed gene of all DRs (I think). GenDRBRev is a reverse primer that anneals to all DR genes and has a SalI for cloning.

PCR was performed with various templates and either BacFlyBFor or LongBfor as a forward primer, and GenDRBrev for reverse. Results were **very inconsistent** with either set of primers, but they did not seem to be temperature sensitive. The conditions I used at the end were 8x(94C,53C,72C) 30x(94C,60C,72C).

In order to amplify some of my reactions, I ran one set of PCR reactions on a gel, picked pieces of agarose and reamplified using just BacFlyBFor and GenDRBrev. I called these reactions 2XPCR reactions.

For Alpha, use S2AlphaFor and NewNewASCrev primers and E601 template and 8x(94C,53C,72C) 30x(94C,60C,72C). These are cloned using BamHI and SalI again.

Primers:

LongDRBfor: contains the entire DR1 SS!
gcggcgtcGGATCCATGGTGTGTCTGAAGCTCCCTGGAGGCTCCTGCATGACAGC
GCTGACAGTGACACTGATGGTGCTGAGCTCCCCACTGGCTTTGGCTGGGGAC
ACCCGACCACGTTTC
BacFlyBFor:
GTGACCCGGGGATCCATGGTGTGTCTGAAGCTCCCTGGAGGCTCCTGCATG
GenDRBrev:
AAGCTTGTCGACGGTACCTCATTACTTGCTCTGTGCAGATTCAGA
NewNewASCrev:
GGGCTAGGTCGACCGTTATCATGGGCATGGAGCATCGAATTCCCAATGCTTG
S2AlphaFor:
GCCGGGTCGGATCCTATAAATATGGCCATAAGTGGAGTCCCTGTG

S2 cell line freezes, TC-XX CELL LINES:

any cell freeze labelled TC-xx is a S2 transfectant.
all made by Tom at various times. stocks are in lN2.

Notes on various S2 transfectants:
All are with pRMHA-3 vector as the expn vector (see Bunch et al Nucl. A Rsrch, 16(3):1043)

Some are double which is Promotor, gene poly A Promotor gene poly A

All are soluble versions of the protein indicated.
Most did not express any protein (as detect by ELISA, limit of detection about 30 ug/L)

In general, the double vector constructs expressed less than the same genes on separate vectors.

Some (DR1SS/*xxxx) have DR1 signalsequence cloned with the extracellular coding of another allele.

Clones:

TC-1	E305	B1*0101 w/ cys, double vector	expresses about 0.2 mg/L
TC-2	E337	*0401	very little expn
TC-3	E346	*0401	very little expn
TC-4	E353	*1501	trace expn?
TC-5	E370	B5*0101	trace expn?
TC-6	E533	B1*13xx	none
TC-7	E537	*1601/2	none
TC-8	E541	*1301/2/6	none
TC-9	E545	*13xx	none
TC-10	E549	*1301/2/6	none
TC-11	E553	*0301	none
TC-12	E557	*1301/2/6	none
TC-13	E561	*0701 NOT!!!!! Has Mut.	none
TC-14	E565	*0301	none
TC-15	E569	*0301kt3	none
TC-16	#196,#197 *0101 no cys, from mellins lab, sep vectors expresses about 10-20 mg/L		
TC-17	E376,#276 *0402kt3 w/ cys, from mellins lab, sep vectors		not tested
TC-18	pNeo alone ----		none

not all of the above are 100% DNA seq.ed. TC-1,2,4,5,16 areall correct. TC-13 has a point mutation in Drbeta.

TC-19	E305	alpha long with ala cys, B1*0101, double vecexpresses low
TC-20	E601,#197	B1*0101 w/ cys sep vecs expresses about 1 mg/L
TC-21	#196,#197	B1*0101 no cys, sep vecs expresses about 2-5 mg/L
TC-22	E601,#285	*0301kt3, w/ cys, sep vecs none
TC-23	E421	*0701 w/cys, not DNA seq.ed, sep vecs, But NOT cuz Mutation in Beta gene
TC-24	E422	*0701 w/cys, not DNA seq.ed, sep vecs, but NOT cuz Mutation in Beta gene
TC-25	E141	*0401 w/cys, sep vecs expresses about 0.5 mg/L
TC-26	#276	*0402kt3 w/cys, sep vecs not tested
TC-27	#282	*0402kt3 w/cys, sep vecs not tested
TC-28	E143	*1501 w/cys, sep vecs trace expn
TC-29	E145	B5*0101 w/cys, sep vecs trace expn
TC-30	#196,#285	B1*0301kt3 no cys, sep vecs non
TC-31	E305	B1*0101 w/ cys double vec Initial screens indicated that
TC-32	E601,#197	B1*0101 w/ cys sep vecs clones TC31-42 expressed very little

TC-33 #196,#197 B1*0101 no cys, sep vecs protein, but lines got contaminated
TC-34 E601,#285 *0301kt3, w/ cys, sep vecs and weren't further assayed.
TC-35 E419 *0701 w/cys, not DNA seq.ed, sep vecs, But NOT cuz Mutation in Beta gene
TC-36 E422 *0701 w/cys, not DNA seq.ed, sep vecs, but NOT cuz Mutation in Beta gene
TC-37 E141 *0401 w/cys, sep vecs
TC-38 #276 *0402kt3 w/cys, sep vecs
TC-39 #282 *0402kt3 w/cys, sep vecs
TC-40 E143 *1501 w/cys, sep vecs
TC-41 E145 B5*0101 w/cys, sep vecs see Elisa
TC-42 #196,#285 B1*0301kt3 no cys, sep vecs NB12-128

					mg/ml
TC 51	#196	#197	Dralpha sol, no cys	B1*0101	0.20
TC 52		#285	Dralpha sol, no cys	B1*0301, with C term kt3	0.03
TC 53		E141	Dralpha sol, no cys	B1*0401	0.70
TC 54		J24	Dralpha sol, no cys	B3*0101	0.30
TC 55		X4-3	Dralpha sol, no cys	DR1SS/B1*0801	0.00
TC 56		X7-3	Dralpha sol, no cys	DR1SS/B5*0101	0.06?
TC 57		X9-1	Dralpha sol, no cys	DR1SS/B1*1501	0.06?
TC 58		X11-2	Dralpha sol, no cys	DR1SS/B1*0301	0.00
TC 59	E601	#197	DRalphaLongAlaCys	B1*0101	2.50
TC 60		X4-3	DRalphaLongAlaCys	DR1SS/B1*0801	0.00
TC 61	X16-3	#197	DRalphashortPCR	B1*0101	5.00
TC 62		#285	DRalphashortPCR	B1*0301, with C term kt3	0.06
TC 63		E141	DRalphashortPCR	B1*0401	0.00
TC 64		J24	DRalphashortPCR	B3*0101	0.07?
TC 65		X4-3	DRalphashortPCR	DR1SS/B1*0801	0.04?
TC 66		X7-3	DRalphashortPCR	DR1SS/B5*0101	0.00
TC 67		X9-1	DRalphashortPCR	DR1SS/B1*1501	0.12
TC 68		X11-2	DRalphashortPCR	DR1SS/B1*0301	0.70

This expn test was on cells in 6 well plates, not carefully controlled for cell density.

Plasmids for transfection of S2 cells:

Alphas	Name	pRMHA-3 with:	Sequencing Status
AlphaSC	X16-3	DRalpha, no connecting peptide, ProCysPro	Perfect
Alpha	#196	DRalpha, full connecting peptide	Perfect
AlphaCys	E601	DRalpha, full connecting peptide, AlaCys.	Perfect

Betas	Name	pRMHA-3 with:	Seq Status
B1*0101	#197	Native SS, native gene with connect. pep.	Perfect
B1*0301	#285	0301SS and gene with conn. pep. And Kt3.	Perfect
B1*0401	E141	Native SS, native gene with connect. pep.	Perfect
B1*0701	E422	Native SS, native gene with connect. pep.	Has a K65E mut in NSQKD
B1*1501	E143	Native SS, native gene with connect. pep.	Perfect
B5*0101	E145	Native SS, native gene with connect. pep.	Perfect
B3*0101	J24	Native SS, native gene with connect. pep.	Perfect
DR1SS/B1*0301	X11-2	DR1SS, silent Arg codon mut, connect. Pep.	Need to confirm end of gene.
DR1SS/B1*0401	X13-1	DR1SS, connecting peptide.	Need to confirm SS.
DR1SS/B1*0701	X15-1	DR1SS, Gln to Arg mut early, con. peptide.	Not done. Has same K65E mut.
DR1SS/B1*0801	X4-3	DR1SS, connecting peptide.	Perfect
DR1SS/B1*1501	X9-1	DR1SS, connecting peptide.	Need to confirm SS.
DR1SS/B5*0101	X7-3	DR1SS, connecting peptide.	Need to confirm SS.

Igalpha/Igbeta and DM plasmid constructs.

The Absolute Master Cheat Sheet.

N101: DMalpha Basic Zipper **N108:** DMbeta Basic Zipper
N113: DMalpha Acid Zipper **N119:** DMbeta Acid Zipper

All Constructs Listed Here have been Sequenced or subcloned from other sequenced constructs (unless it says "should be").

All Constructs are with Murine Igalpha (mb-1 gene) and Murine IgBeta (b29 gene).
Sequence Listed is from Sequencing Reactions, so this is really what is there!

CDNA: see NB-1 p. 1 for-info from Jeff Schneringer in John Cambier's Lab. mb-1 cDNA is in pBluescript SK- via BamHI 5' and Xhol 3'. Note that published sequence is wrong. There is an inserted C (282) and a deleted G (300) relative to GenBank M31773. It is confirmed that this IS the correct cDNA because there exist later mb-1 sequence reports that confirm it and it matches better with other Ig domains in the family. B29 cDNA is cloned into EcoRI sit of pSK. See Hermanson PNAS 85('88):6890 for sequence.

E. Coli Cytoplasmic Expn:

pLMI vectors start gaattcaggaggaatttaaaATGgene

N92: IgAlpha-basel-cyto (often referred to as Alpha Zip) in pLMI
LRVE ... EKPStpStpHindIIIpLMI
tta cgc gta gaa ... gaa aag cca taa tga aag ctt

N142 IgAlpha-basel. (IgalphaZipStop or IgalphabaseStop).
LRVE ... KLAQStpStpHindIIIpLMI
tta cgc gta gaa ... etc gcc cag tga taa get tgg ctg ttt tgg

N131 IgAlpha-Acidl. (Igalphaacidstop) . (Xba->ATG=pET32, rest=pLMI)
subcloned from N99 Xba/BspEl N92 XbaBspEICip

N29: IgAlpha-HisTag. (no zip or cyto). in Pet25b
HMLRVE ... KNRSGHHHHHHAOchEcoEagPet25b
cat atg tta cgc gta gaa ... aag aac egg tec gga cat cac cac cat
cac cat get taa gaa ttc egg ccg

M72: IgBeta-acidl-cyto (often referred to as BetaZip). in Pet32a
HMSSDL ... PGQEOpaEcoSalHindEagXhoHHHHHH PET32a
cat atg age agt gac ctg ... cca ggc cag gaa tga gaa ttc gag etc
cgt cga caa get tgc ggc cgc act cga gca cca cca ...

M63: IgBeta-STag (no zip or cyto)
HMSSDL ... MDSOchOpaEagXhoHHHHH pET32a
cat atg age agt gac ctg ... atg gac age taa tga egg ccg cac teg
age ace ace

M67: IgBeta-BTag (no zip or cyto) in Pet32a
HMSSDL ... PGQNOpaAmbEcoEagXhoHHHHHH
cat atg age agt gac ctg ... cag gaa tga tag gaa ttc gcg gcc gca

N144: IgBeta-basel. (IgbetaBaseStop). N98: same as N144.
HMSSDL ... KLAQStpStpHindIIIpLMI
cat atg age agt gac ctg ... etc gcc cag tga taa get tgg ctg ttt tgg

N99: IgBeta-Acidl. (Igbetazipstop). (Xba->ATG=pET32, rest=pLMI)
HMSSDL ... LAQStpStpHindIIIpLMI
cat atg age agt gac ctg ... ctg get cag tga taa get tgg ctg ttt

N35: IgAlpha-HisTag with one Cys->Ser (RSC).
identical to N29 start and end.

B'123-2: IgBeta-BTag with three cys->ser (SPC, QKC, DSC). in Pet25b
HMSSDL ... PGQNOpaAmbEcoEagXhoHHHHHH

cat atg age agt gac ctg ... cag gaa tga tag gaa ttc gcg gcc gca

N16-21: should all be IgBeta-BTag with first two C->S but not third.

N22-27: should all be IgBeta-BTag with third C->S but not 1st or 2nd.

E. Coli Periplasmic Expn:

All constructs (PET27b) ... HMKYLLPTAAAGLLLLAAQPAMA/MDIGINSVPAMT-
PelB Linker Gene Ndel Ncol

M390: PelB-Igalpha-Histag in pPet32a.
LRVE ... KNRSGHHHHHHAOchEcoEagXhoHHHHHH
tta cgc gta gaa ... aag aac egg tec gga cat cac cac cat cac cat
get taa gaa ttc egg ccg cac teg age ace ...

M398: PelB-Igalpha-Histag in pPet25b subcloned from M390 via NdeEag.

M358: PelB-Igalpha-basel-cyto in pPet27b (Kan resistance!)

M368: PelB-Igalpha-basel-cyto in pPet25b subcloned (M358) via Nde/Eag.
LRVE ... LEKPOpaEcoAflIIEagXhoPet25b
tta cgc gta gaa ... ctg gaa aag cca tga gaa ttc cct agg gcg

M61: PelB-Beta-Stag in Pet32.
SSDL ... MDSOchOpaEagXhoHHHHHPet32
age agt gac ctg ... atg gac age taa tga egg ccg cac teg age ace

M291: PelB-Beta-Btag in Pet32.
SSDL ... PGQEOpaAmbEcoEagXhoPet32a
age agt gac ctg ... ggt cag gaa tga tag gaa ttc gcg gcc gca etc
gag cac cac ...

M77: PelB-IgBeta-acidl-cyto in Pet32a.
SSDL ... PGQEOpaEcoSacSalHindEagXhoPet32 ...
age agt gac ctg ... ggc cag gaa tga gaa ttc gag etc ...

M408: PelB-Igalpha-Histag PelB-IgBeta-Stag.
Vector: Sph/Xba/Cip M61 Insert: Sph/Nhe M398

M411: PelB-Igalpha-Histag PelB-IgBeta-Btag.
Vector: Sph/Xba/Cip M291 Insert: Sph/Nhe M398

M393: PelB-Igalpha-basel-cyto PelB-Igbeta-Acidl-cyto.
Vector: Sph/Xba/Cip M77 Insert: Sph/Nhe M368

Pichia vectors:

These are all clones Xhol to EagI with the indicated joints.

M346: Pic9K Igalpha-Histag subcloned from M79 AatII-XbaI.

M79: Pic9 Igalpha-Histag.
(LEKREAEALRVE .... NRSGHHHHHHAtaaEcoRIEagI)
(gaa get tta cgc gta gaa ... cat get taa gaa ttc egg ccg cga att).

M371: Pic9KIgalpha-basel-cyto subcloned from M358 MluI-EagI.
(LEKREAEALRVE ... EKPtaaEcoRI)
(get gaa get tta cgc gta gaa ... subcloned MluI-EagI from M358)

M355: Pic9KIgbeta-Btag subcloned from M85 AatII/XbaI.

M85: Pic9 Igbeta-BTag.
(LEKREAEASSDL ... PGQEOpaAmbEcoEag)
(gaa get age agt gac ... cag gaa tga tag gaa ttc gcg gcc gcg aat taa)

M349: Pic9K Igbeta-acidl-cyto subcloned from M83 AatII/XbaI.

M83: Pic9 Igbeta-acidl-cyto.
(LEKREAEASSDL ... PGQEOpaEcoSalHindEag
(gaa get age agt gac ... cag gaa tga gaa ttc gag etc (c)gt cga caa
get tgc gga cgc gaa tt)

See Mia 12/97 Friday Meeting for DM cloning info.

BTag:
tec gga gaa gtt aaa tgg tec gtt ggt gaa cac ccg ggt cag gaa
Ser Gly Glu Val Lys Trp Ser Val Gly Glu His Pro Gly Gin Glu Stop

STag:
tec gga aaa gaa ace get get get aaa ttc gaa cgc cag cac atg gac age
Ser Gly Lys Glu Thr Ala Ala Ala Lys Phe Glu Arg Gin His Met Asp Ser Stop

pAcid1 zipper
DNA
TCCGGAGCTCAGCTCGAAAAAGAGCTCCAGGCCCTGGAGA
AGGAAAATGCACAGCTGGAATGGGAGTTGCAAGCACTGGA
AAAGGAA.CTGGCTCAG.GGATCC
Protein
SGAQLEKELQALEKENAQLEWELQALEKELAQGS

pBase1 zipper
DNA
tccggaGCTCAGTTGAAAAAGAAATTGCAAGCACTGAAGA
AAAAGAACGCTCAGCTGAAGTGGAAACTTCAAGCCCTCAA
GAAGAAACTCGCCCAGggatcc
Protein
SGAQLKKKLQALKKKNAQLKWKLQALKKKLAQGS

MurineIgAlphaExtracellu
lar
DNA
ttaCGcGTAGAAGGGGGTCCACCATCCCTGACGGTGAACT
TGGGCGAGGAGGCCCGCCTCACCTGTGAAAACAATGGCAG
GAACCCTAATATCACATGGTGGTTCAGCCTTCAGTCTAAC
ATCACATGGCCCCCAGTGCCACTGGGTCCTGGCCAGGGTA
CCACAGGCCAGCTGTTCTTCCCCGAAGTAAACAAGAACCA
CAGGGGCTTGTACTGGTGCCAAGTGATAGAAAACAACATA
TTAAAACGCTCCTGTGGTACTTACCTCCGCGTGCGCAATC
CAGTCCCTAGGCCCTTCCTGGACATGGGGGAAGGTACCAA
GAACCGg

Protein
LRVEGGPPSLTVNLGEEARLTCENNGRNPNIT
WWFSLQSN
ITWPPVPLGPGQGTTGQLFFPEVNKNHRGLYW
CQVIENNI
LKRSCGTYLRVRNPVPRPFLDMGEGTKNR

Murinelgalpha cytoplasmic

DNA
AGGAAgCGcTGGCAAAATGAGAAGTTTGGGGTGGACATGC
CAGATGACTATGAAGATGAAAATCTCTATGAGGGCCTGAA
CCTTGATGACTGTTCTATGTATGAGGACATCTCCAGGGGA
CTCCAGGGCACCTACCAGGATGTGGGCAACCTCCACATTG
GAGATGCCCAGCTGGAAAAGCCA

Protein
RKRWQNEKFGVDMPDDYEDENLYEGLNLDDCSMYEDISRG
LQGTYQDVGNLHIGDAQLEKP

Murinelgbeta Extracellular

DNA
AGCAGTGACCTGCCACTGAATTTCCAAGGAAGCCCTTGTT
CCCAGATCTGGCAGCACCCGAGGTTTGCAGCCAAAAAGCG
GAGCTCCATGGTGAAGTTTCACTGCTACACAAACCACTCA
GGTGCACTGACCTGGTTCCGAAAGCGAGGGAGCCAGCAGC
CCCAGGAACTGGTCTCAGAAGAGGGACGCATTGTGCAGAC
CCAGAATGGCTCTGTCTACACCCTCACTATCCAAAACATC
CAGTACGAGGATAATGGTATCTACTTCTGCAAGCAGAAAT
GTGACAGCGCCAACCATAATGTCACCGACAGCTGTGGCAC
GGAACTTCTAGTCTTAGGATTCAGCACGTTGGACCAACTG
AAGCGGCGGAACACACTTAAGGAT

Protein
SSDLPLNFQGSPCSQIWQHPRFAAKKRSSMVKFHCYTNHS
GALTWFRKRGSQQPQELVSEEGRIVQTQNGSVYTLTIQNI
QYEDNGIYFCKQKCDSANHNVTDSCGTELLVLGFSTLDQL
KRRNTLKD

MurineIgBeta cytoplasmic

DNA
GACAAGGATGACGGCAAGGCTGGGATGGAGGAAGATCACA
CCTATGAGGGCTTGAACATTGACCAGACAGCCACCTATGA
AGACATAGTGACTCTTCGGACAGGGGAGGTAAAGTGGTCG
GTAGGAGAGCATCCAGGCCAGGAA

Protein
DKDDGKAGMEEDHTYEGLNIDQTATYEDIVTLRTGEVKWS
VGEHPGQE

T cell Clones and Lines:

Patients:

1H, is a DR1-homozygote in Rafick Sekaly's lab. I received a bleed of 50 ml in K3EDTA vacutainers fedex o/n from Bader on 9/13/00, and got 160 million PBMCs. Froze 110 million, and stimulated 50.

SL08, is a DR1+ volunteer in the Stern lab from the Influenza study. I have gotten blood from this volunteer on numerous occasions, usually 50-100 ml.

SL07, is a DR4 homozygote (really? I think so) from the Stern lab Influenza study. I have gotten blood from this volunteer several times.

My New Lines and Clones:

See NB12-132 for info on receipt of the 1H blood and its workup, NB13-11 for sorting notes.

1H FB: refers to polyclonal line derived from initial stimulation of fresh 1H PBMCs on 9/13/00. Further peptide stimulations: added IL-2 on 9/20, 9/24, new stim 9/27/00, IL-2 9/28, and continuing with IL-2 until 10/17/00 when I did a round of sorting and restimulated the non-stained cells with peptide. Further stimulations 11/14 and 12/6.

1H HA: refers to polyclonal line derived from initial stimulation of fresh 1H PBMCs on 9/13/00. Further peptide stimulations: added IL-2 on 9/20, 9/24, new stim 9/27/00, IL-2 9/28, and continuing with IL-2 until 10/17/00 when I did a round of sorting and restimulated the non-stained cells with peptide. Further stimulations 11/14 and 12/6. Jen Cochran did significant work cells from this line.

1H HA Tet+ CFSEhigh: cells from 1H HA that were sorted on 10/17/00 with DR1HA tet+ and CFSE high phenotype. They were stimulated with PHA/IL2 but never grew.

1H HA Tet+: cells from 1H HA that were sorted on 10/17/00 with DR1HA tet+ and CFSE low phenotype. They were stimulated with PHA/IL2 10/17, 11/3, and with HA peptide 11/14 and 12/6.

1H HA Tet-: cells from 1H HA that were sorted on 10/17/00 with DR1HA tet- and CFSE low phenotype. They were stimulated with PHA/IL2 10/17, 11/3, and with HA peptide 11/14 and 12/6.

1HFB01-1HFB34, 1HHA01-1HHA07: clones derived on 10/17 by single cell cloning of 1H FB or 1H HA based only on CFSElow phenotype (no tetramer staining). Plates were stimulated 10/17 and 11/3, and only after the second stimulation were some plugs of growing clones visible to the eye. Originally 180 wells of 1H FB were sorted, and 300 wells of 1H HA, and only these 34/7 wells grew sizable plugs. Cells were transferred to flat bottom wells for further culturing with IL-2. On 11/14, cells were split and half were stimulated with PHA/IL2 and half with peptide (FluB or HA) and IL-2. I later observed that for all clones, it appeared that the peptide stimulation worked better. 11/17 I did a small prolif test on each clone (see NB13-23). 11/21 the cells on the both plates were split into adjacent wells. On 11/30 one of the pep-stim wells, and both of the PHA-stim wells were pooled and each clone was frozen seperately in a small volume of freezing media.

On 12/6, the remaining flat-bottom well of each clone was split into 12 rnd-bottom wells and stimulated with peptide (FluB or HA). At this point, I think that most of these are dead. But there may be some freezes in lN2 with enough cells to stimulate. But, they all seemed to poop out in culture very quickly after being "cloned".

SL08 HA, SL07 HA: On 10/3/00 Mia obtained blood from these volunteers for a DC expt, and I snagged about 10 million PBMCs from her of each. Stimulated with HA peptide 10/3, started IL-2 on 10/10 and continued IL-2 until I restimulated them 11/3. I first stained them on Oct 24 and saw nice staining (even for SL07 who is DR4!). They grew very quickly, and on 11/14 I froze all of them down.

1H HA2, 1H FB2: Oct 24 I thawed some 1H cells and stimulated with HA and FluB peptide. I passaged them further with IL2 and still haven't restimulated them. I plan on doing some sorting this week of these cells. I stained 1H HA2 on 12/9 and it looked good.

SL08 HA 2, KH HA, ST HA: Nov 28, 00 I obtained 50 ml from SL08 and got 90 million PBMCs. George Cohen got blood from KH and ST and received 60 and 10 million respectively. I froze some of the SL08 blood, used some of each patient for tet staining and IFNg analysis that day, and stimulated the rest with HA peptide. Added IL-2 on days 5, 9, 13. On Day 11 I stained some SL08HA2 and KHHA and saw good signal (around 5% tet+). I plan on using these for more staining, sorting, and IFNgamma analysis this week.

<u>Other Clones/Lines, not derived by me:</u>

HA1.7: classic DR1HA specific clone from Jonathan Lamb. Jen Cochran has done much of her work on this line.

Cl-1: another DR1HA specific clone, this time from A. Sette. Cross reacts with DR4, I think.

HA03: polyclonal line derived from 1H and grown by Bader Yassine-Diab. He grows lines quite differently: he depleted CD8s from the PBMCs and stimulated with HA every seven days with autologous PBMCs for several weeks before he sent the line to me. I passaged it bi-weekly (or less frequently) as I do all the lines. In my hands this has always been a long-term line. I have many freezes of this line from various times. I used this line in my JI Jan 2001 manuscript, last figure.

HaCoH8, HaCoH2: Last summer, Jenny and I sent reagents to Corinne Moulon at Warner-Lambert in France to try to stain Ni-reactive T cell clones that she studied. We sent her some DR1HA as a control. She tested these two lines which she had derived, HaCoH8 and HaCoH2. I sometimes call them H8 and H2. H8 stained at both 37C and 4C. H2 at neither. She grows these cells with six-week rests between each stimulation! I don't. **Neither seem to grow very well.** I think H2 likes high doses of HA and both seem to like stimulation in rnd bottom wells.

Peptide that I have used.

Name	Sequence	Synth Pred.	MW	Ret. Time
p24(8)	GQMVHQAISPRTL	mVh tough	1435 MW,	HPLC 15.67
p24(34)	PEVIPMFSALSEG	OK	1374 MW	HPLC 23.3
p24(37)	IPMFSALSEGATP	OK	1317 MW	HPLC 21.42
p24(31)	AFSPEVIPMFSA	OK	1293 MW	HPLC 24.4
p24(84)	HPVHAGPIAPGQM	good	1309 MW	HPLC 13.4
p24(89)	GPIAPGQMREPRG	v. good	1363 MW	HPLC 11.73
p51(169)	EPFRKQNPDIVIY	OK (DIVI hard)	1618 MW	HPLC 19.38
P51(177)	DIVIYQYMDDLYV	DIVI hard	1649 MW	HPLC 29.12
p51(269)	QIYPGIKVRQLCK	OK	1542 MW	HPLC 18.09
p51(272)	PGIKVRQLCKLLR	OK	1520 MW	HPLC 20.08
gag	PIVQNIQGQMVHQAIS	1763 MW		
Nef	LAFHHMARELH	1361 MW		
Nef 70-80	VGFPVRPQVPL	1208 MW	21.32 HPLC, easy synth.	

TT QYIKANSKFIGITEL MW 1725
TT QYIKANSKFIGITE MW 1611. This is the one that I have.
OspA SYVLEGTLTAEKTTLVVKEG
LFA-1 ELQKKIYVIEGTSKQDLTSF
P51(324) EPFRKQNPDIVIYQY
P51(331) PDIVIYQYMDDLYVG
P24(133) PIVQNIQGQMVHQAIS
PIVQNIQGQMVHQAIS
P24(166) PEVIPMFSALSEGAT
PEVIPMFSALSEGAT
HA (306-318) PKYVKQNTLKLAT MW 1503
Ha307R PRYVKWNTLKLAT in Sydney influenza strains (A strains only) (from (hemaglutinin) Water Sol
A2 [103-114] VGSDWRFLRGYHQYA MW1855 Water Sol.
NP [206-217] NFWRGENGRKTRN in nearly all influenza viruses (from nuccleoprotein) Water sol.
TfR [680-696] RVEYHFLSPYVSPKESP MW 2034, Water Sol
FluB PYYTGEHAKAIGN in Ann Arbor influenza strains (B strains only) (from hemaglutinin) Water Sol
MA SGPLKAEIAQRLED MW 1526 in nearly all influenza strains (from matrix protein) Water Sol

P5 peptide GDLLGILESRGIKAR (measles). MW 1596.

HBV-112 RETV**L**EY**L**V**S**FG**V**W mw 1698, PREDICT 29.5 hplc RETENTION (PRETTY LONG), difficult synth., Net –1 charge, but multiple of each-
I made this peptide with a free amine at NH end, and an Amide at Cterm., Sol in DMSO

HBV-116 **L**EY**L**V**S**FG**V**WIRTP mw 1680, predict 31.25 hplc, retention (pretty long), medium difficult synth., net nuetral charge, 2 of each.
HBV-116-2 amino-GE**Y**LV**S**F**G**VW**I**RTP-acid, MW 1624.
HBV-116-3 amino- GE**Y**LV**S**F**G**VW**I**RTamide, MW 1526 only sol. In DMSO
HBV-122 FGV*WI*R*TP*P*A*Y*RP*PN mw 1771, predict 22.8 (pretty early), easy synthesis, Net +2 charge, 3+ and the carboxy terminus.

p24(131) amino-KRWIILGLNKIVR-acid 1609 MW, Predict 25.63 HPLC, difficult synthesis, net +4 charge, sol in 50/50 H2O/CAN, or 6M GuHCl
p24(140) amino-KIVRMYSPTSIL-acid 1408 MW, Predict 21.42 HPLC, easy synth, net +2 charge.

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EDUCATION

Massachusetts Institute of Technology, Cambridge, MA, Ph.D., Department of Chemistry, February 2002
Cornell University, College of Arts and Sciences, Ithaca, NY, *Summa cum laude,* B.A., Department of Chemistry, May 1994

HONORS & AWARDS

NIH Biotechnology Training Grant fellowship, Sep. 98 – Dec. 01
Summa cum laude, Chemistry, Cornell University, May 94
George Caldwell Prize in Chemistry, Cornell University, May 94
Phi Beta Kappa, Cornell University, May 94
Dean's List, Cornell University, Fall and Spring semesters, 90 thru 94.

RESEARCH EXPERIENCE

Research Fellow, *Jan. 95 - Jan. 02,* Prof. Lawrence Stern, Department of Chemistry, MIT
Investigating structure and function of the T-cell receptor with soluble oligomers of its ligand, class II Major Histocompatibility Complex.

Research Assistant, *Sept. 92 - Aug. 94,* Prof. Barbara Baird, Department of Chemistry, Cornell University
Studied the transmembrane signaling mechanism of FcεRI receptor on mast cells using dansyl ligands.

Research Assistant, *Summer 92*, Prof. Lynmarie Thompson, Department of Chemistry, Univ. of Mass.
Studied the cytoplasmic methylation of the *E. coli* Aspartate Receptor.

Research Assistant, *Summer 91*, Prof. Sharon Palmer, Department of Chemistry, Smith College
Investigated the synthesis and electrochemistry of Iron (III) tetra-carboxy tetraphenylporphyrin.

PUBLICATIONS

Zaru, R., Cameron, T. O., Stern, L. J., Shaw, A., Muller, S. and S. Valitutti. TCR Engagement and Triggering in the Absence of large Scale Molecular Segregation at the T cell-APC Contact Site. *manuscript in preparation.*

Cameron, T. O., Stern, L. J., and G. B. Cohen. Towards TCR Proteomics: Examination of a highly diverse repertoire of $CD4^+$ T cells specific for an influenza peptide bound to HLA-DR1. *manuscript in preparation.*

Cameron, T. O., Norris, P. J., Patel, A., Moulon, C., Rosenberg, E. S., Mellins, E. D., Wedderburn, L. R. and L. J. Stern. Labeling antigen-specific $CD4^+$ T cells with class II MHColigomers. *J. Imm. Methods.* submitted.

Cameron, T. O., Stone, J. D., Cochran, J. R. and L. J. Stern. TCR losing its inhibitions. *Trends in Immunology* 2001. 22(9):479-80.

Cochran, J. R., Stone, J. D., Cameron, T. O., Lubetsky, J.B. and L. J. Stern. Receptor proximity, not intermolecular orientation, is critical for triggering T cell activation. *J Biol Chem.* 2001 276(30):28068-74.

Cochran, J. R., Aivazian, D. A., Cameron, T. O. and L. J. Stern. Receptor clustering and transmembrane signaling in T cells. Review. *Trends Biochem Sci.* 2001 26(5):304-10.

Cameron, T. O., Cochran, J. R., Yassine-Diab, B., Sekaly, R.-P. and L. J. Stern. Detection of antigen-specific $CD4^+$ T cells by HLA-DR1 oligomers is dependent on an active cellular response. *J. Immunol.* 2001. 166(2):741-745.

Cochran, J. R., Cameron, T. O. and L. J. Stern. The relationship of MHC-peptide binding and T cell activation probed using chemically defined MHC class II oligomers. *Immunity.* 2000. 12(3):241-50.