

Review

## Labeling antigen-specific CD4<sup>+</sup> T cells with class II MHC oligomers

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### Abstract

Class I MHC–peptide oligomers (MHC tetramers) have become popular reagents for the detection and characterization of antigen-specific CD8<sup>+</sup> T cells. Class II MHC proteins can be produced by expression in *Escherichia 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. 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<sup>+</sup> T cells. We explore the experimental conditions necessary for efficient staining of CD4<sup>+</sup> 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.

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**Keywords:** MHC tetramers; Helper T cell; T lymphocyte; Avidity; Receptor–ligand interactions; Multi-valent binding; Flow cytometry; Binding assay; HLA-DR

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**Abbreviations:** MHC, major histocompatibility complex; DR1, HLA-DR1; PBMC, peripheral blood mononuclear cells; BSP, biotin–ligase substrate peptide; SA, streptavidin; PE, R-phycoerythrin; FITC, fluorescein isothiocyanate; CFSE, carboxyfluorescein diacetate succinimide ester; SA–PE, PE-conjugated SA; SA–FITC, FITC-conjugated SA; SA–Alexa, Alexa-488-conjugated SA; DTT, 1,4-dithiothreitol.

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### 1. 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 routinized. 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 et al. (1996), has enabled the direct identification of antigen-specific CD8<sup>+</sup> 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<sup>+</sup> T cell populations (Crawford et al., 1998; Novak et al., 1999; Savage et al., 1999; Kwok et al., 2000; Meyer et al., 2000; Cameron et al., 2001). Some of these results indicate that the application of MHC tetramer technology to CD4<sup>+</sup> T cells may not be straightforward. In particular, CD4<sup>+</sup> 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<sup>+</sup> T cells often are too small to be analyzed without amplification in vitro (Kwok et al., 2000). These barriers to the routine use of MHC tetramer staining, while present to some extent in the analysis of CD8<sup>+</sup> T cells, appear to be more formidable in the analysis of CD4<sup>+</sup> 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<sup>+</sup> T cell clones, polyclonal lines, and samples of peripheral blood. We describe the varying temperature requirements for staining CD4<sup>+</sup> 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.

## 2. Materials and methods

### 2.1. Peptides

Peptides Ha[306–318] (PKYVKQNTLKLAT), FluB[308–320] (PYYTGEHAKAIGN), p24(34)[34–46] (PEVIPMFSALSEG), A2[103–114] (VGSDWRF LRGYHQYA) and Tfr [680–696] (RVEYHFLSPYV SPKESP) 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).

### 2.2. Antibodies and streptavidin

Mouse monoclonal antibody OKT4 (anti-human CD4) (ATCC, Manassas, VA) was purified from hybridoma supernatant by protein-A Sepharose (Repligen, Needham, MA). For fluorescent labeling, antibody or streptavidin (Prozyme, San Leandro, CA) was incubated with 10-fold molar excess FITC (Sigma-Aldrich, St. Louis, MO) in 10% DMSO, or Alexa-488 succinimide ester (Molecular Probes, Eugene, OR) in aqueous solution, for 3 h 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-hydroxyazobenzene-4'-carboxylic acid (Haba, Pierce Chemical) 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, 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<sub>H</sub>0 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, Norway.

### 2.3. DR subunit bacterial expression

Soluble HLA-DR1 was produced by expression of individual subunits in *Escherichia 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 ( $\alpha$ 1–190,  $\beta$ 1–192) 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 $\alpha_{\text{cys}}$ ) (Cochran and Stern, in press) or C-terminal biotin-ligase substrate peptide tag (Schatz, 1993) (GSLHHILDAQKMV-WNHR) (DR $\alpha_{\text{BSP}}$ , a generous gift of Souheil Younes and Rafick Pierre Sekaly, University of Montreal). BL21 DE3 *E. coli* were transformed with either pLMI DR $\alpha$  or pLMI DR $\beta$  plasmids and stored as glycerol stocks at  $-70^{\circ}\text{C}$ . Overnight cultures were used to seed 1–10 l of Luria Broth supplemented with 50  $\mu\text{g}/\text{ml}$  ampicillin and 0.2% D-glucose, induced at OD<sub>600</sub> 1.0–1.5 with 0.5 mM IPTG, and harvested 3 to 5 h later.

### 2.4. Isolation of crude inclusion bodies

Inclusion bodies containing DR subunits were isolated using a modified detergent extraction protocol (Nagai and Thogersen, 1987). *E. coli* cell pellets from 10 l 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 10 min, 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<sub>2</sub>, 10 mM DTT). One milligram of DNase I was added, the solution was stirred until its viscosity was reduced significantly (10–20 min), and then frozen at  $-20^{\circ}\text{C}$ . Solutions were later thawed, centrifuged at  $6000 \times g$ , 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  $-70^{\circ}\text{C}$ .

### 2.5. Ion exchange purification of DR inclusion bodies

Crude inclusion body pellets were thawed, centrifuged at  $6000 \times g$  to remove insoluble material, filtered through 0.45- $\mu\text{m}$  membranes, and treated with additional 30–50 mM DTT for 10–20 min at room temperature to ensure complete reduction of cysteines. On a Perceptive 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  $\alpha$ ) or 9.0 (for  $\beta$ ), 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  $-70^{\circ}\text{C}$ .

### 2.6. 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  $\mu\text{M}$  peptide was chilled and purified inclusion bodies were added dropwise to the stirring buffer to a final concentration of 10 mg/l of each subunit. Reactions were incubated at  $4^{\circ}\text{C}$  for 2 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 10 min, 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 h at 4 °C. Alternatively, the filtrate can be exchanged into the same buffer by tangential ultrafiltration using a Pelli-con 10k cartridge (Millipore, Bedford, MA). The buffer-exchanged sample was centrifuged at  $6000 \times g$  to remove insoluble material, and filtered through 0.45- $\mu$ m 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 min 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<sub>cys</sub> 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/l of folding mix.

### 2.7. Chemical biotinylation of DR1<sub>cys</sub>

For biotinylation of DR1<sub>cys</sub> constructs, DR-containing fractions were pooled, freshly dissolved biotinyl-3-maleimidopropionabmidly-3,6-dioxaoctanediamine (PEO-maleimide–biotin, Pierce Chemical) was added to a final concentration of 50  $\mu$ M, and the mixture was incubated for 10–30 min 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) and purified on a gel filtration column (SEC-3000, Phenomenex, Torrance, CA) in phosphate buffered saline (PBS, 15 mM Na/K PO<sub>4</sub>, 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 12 months when stored at 4 °C.

### 2.8. Enzymatic biotinylation of DR1<sub>BSP</sub>

As an alternative to chemical biotinylation, a C-terminal BSP tag can be enzymatically biotinylated using biotin-ligase (Avidity, Denver, CO) (Schatz, 1993). DR1<sub>BSP</sub> was prepared as described for DR1<sub>cys</sub>. 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  $\mu$ M biotin), supplemented with 5  $\mu$ g/ml biotin ligase (Avidity), and incubated 30 min 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.

### 2.9. DR1<sub>cys</sub> expression in insect cells

Expression of DR1<sub>cys</sub> in insect cells was initially performed using baculoviral infection of Sf9 cells essentially as described (Stern and Wiley, 1992). Subsequent expression using stable transfection of S2 Schneider 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 DR $\beta$  (1–192) were generous gifts from Elizabeth Mellins (Stanford University). DR $\alpha$ (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 DR $\alpha$ <sub>cys</sub> (1  $\mu$ g), pRMHa-3 DR $\beta$  (1  $\mu$ g), and pNeo (0.05  $\mu$ g) 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 4 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  $\mu$ g/ml streptomycin (Gibco), 250  $\mu$ g/ml 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  $5-10 \times 10^6$  per ml by addition of 0.5 mM  $\text{CuSO}_4$ , and culture supernatant was collected 4 to 6 days later by centrifugation at  $4000 \times g$ . Supernatant was concentrated 10-fold in a 10,000 molecular weight cutoff spiral filtration device (Millipore).  $\text{DR1}_{\text{cys}}$  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  $\mu\text{M}$ , and the mixtures were reacted at room temperature for 20–30 min 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 37 °C in the presence of an appropriate concentration of peptide (usually three- to fivefold 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 nondenaturing 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 4 °C. Final yield was approximately 0.1–0.3 mg DR1-peptide-biotin per liter S2 cell culture.

### 2.10. 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 min 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  $\epsilon_{280}$  values of 18,800  $\text{M}^{-1}$  for SA, 15,200  $\text{M}^{-1}$  for FITC, 8000  $\text{M}^{-1}$

for Alexa-488, and  $\epsilon_{490}$  values of 69,000  $\text{M}^{-1}$  for FITC and 78,000  $\text{M}^{-1}$  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  $\mu\text{m}$  spin filters (Costar) before analysis. Molecular weight equivalents (Da) were calculated from hydrodynamic radii (nm) using a standard curve model for globular proteins,  $\text{MW} = (1.549 \times R_{\text{H}})^{2.426}$ .

### 2.11. T cell clones and lines

T cell clones HA1.7 (Lamb et al., 1982), CI-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  $\mu\text{g}/\text{ml}$  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 nonautologous peripheral blood lymphocytes and a DRB1\*0101 EBV-transformed B cell line (EBV1.24) that had been pulsed with 1  $\mu\text{M}$  Ha peptide. Every 3–4 days, 40 units/ml interleukin-2 (Aldesleukin, Chiron, Emeryville, CA) was added to the cultures.

Short-term polyclonal T cell lines were raised by *in vitro* stimulation of lymphocytes isolated from DR1<sup>+</sup> volunteers. Freshly isolated PBMCs were first labeled with 1  $\mu\text{M}$  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  $\mu\text{M}$  peptide.

On days 7 and 10 after stimulation, 40 units/ml IL-2 was added. Secondary stimulation on day 14 with 5  $\mu$ M peptide and nonautologous PBMCs and DR1<sup>+</sup> EBV-transformed B cells was performed as described 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<sup>+</sup> PBMCs were stimulated with 7  $\mu$ M Ha peptide in media, and supplemented on day 7 with IL-2 (100 units/ml). On day 12, T cells 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  $\mu$ g/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<sup>+</sup> subject AC-25 was derived via limiting dilution. Freshly isolated PBMC ( $1 \times 10^7$ ) were suspended in 10 ml of media in a T25 flask and stimulated with p24 (1  $\mu$ g/ml, Protein Sciences, Meriden, CT) and IL-2 (100 units/ml, Hoffman La Roche). For the first 4 weeks of culture, the media was supplemented with Indinavir (Merck, 0.4  $\mu$ M), AZT (Glaxo Wellcome, 0.5  $\mu$ M), and 3TC (Glaxo Wellcome, 3  $\mu$ M) to block HIV replication. After 2 weeks, the PBMC were restimulated with p24 protein (1  $\mu$ g/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  $\mu$ g/ml hygromycin, 4  $\mu$ g/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.

### 2.12. 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  $\mu$ g/ml oligomer, in 10–20  $\mu$ l 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 h. Higher concentrations of oligomer and longer staining times have been shown to result in brighter staining (Cameron et al., 2001), but were not 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 5 min, and stained with CD4-APC for 20–30 min 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.

## 3. Results

### 3.1. 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 homogeneously with a peptide of choice. To produce homogeneously 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. 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

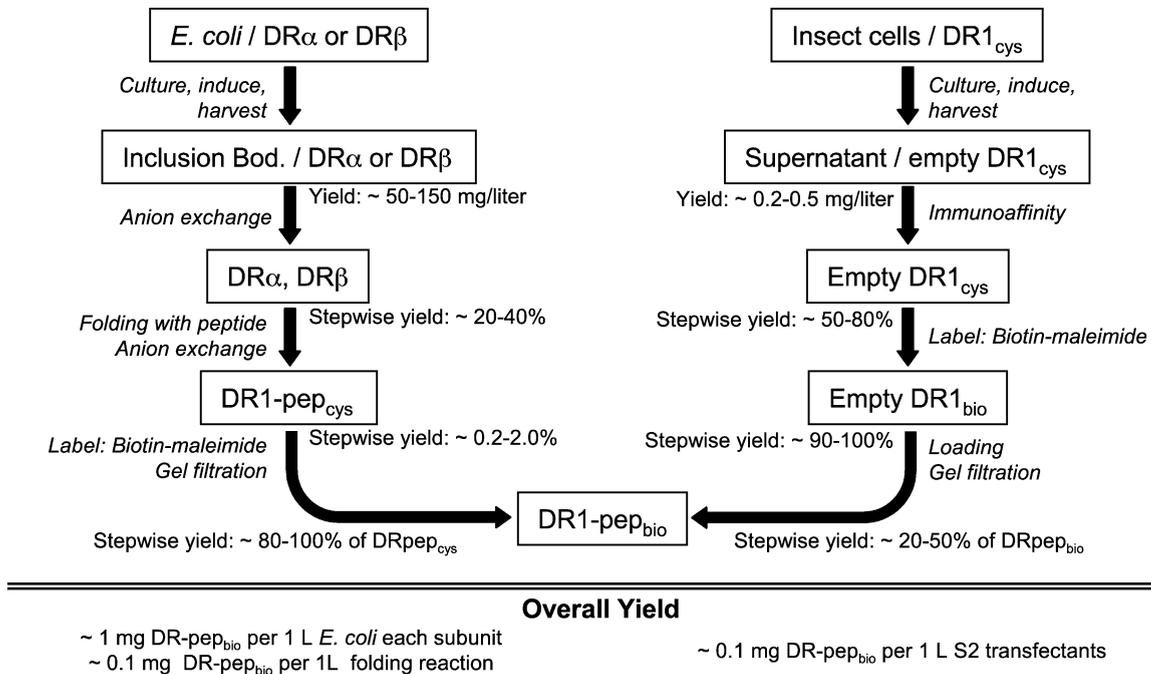


Fig. 1. Production of biotinylated class II MHC. DR1 can be produced either from *E. coli* (left panel) or insect cells (Sf9/baculovirus, or S2, right panel). Yield of MHC protein and experimental effort required are similar in each system. Biotinylation can be achieved enzymatically using BSP and biotin-ligase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

insect-cell peptides using high concentrations of exogenous peptide (Fig. 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 DR $\alpha$ <sub>cys</sub>, DR $\alpha$ <sub>BSP</sub>, and DR $\beta$  subunits were expressed in *E. coli* using methods developed for the unmodified protein (Frayser et al., 1999). Fig. 2a shows SDS-PAGE analysis of total cell lysates before and after induction of DR subunits (NI and I, respec-

tively). The induced  $\alpha$  and  $\beta$  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  $\alpha$  and  $\beta$  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 and Wiley, 1992). The folded DR-peptide complexes in Fig. 2b migrate as

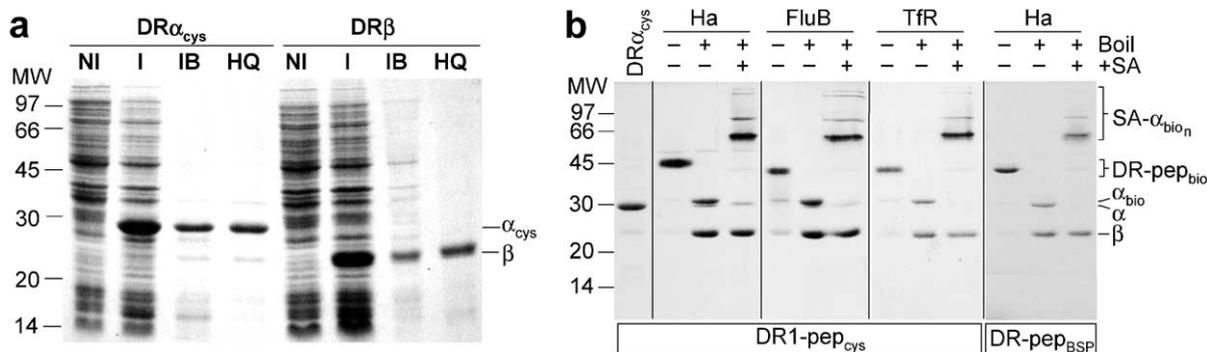


Fig. 2. SDS-PAGE analysis of class II MHC expression, purification, folding, and biotinylation. (a) SDS-PAGE of *E. coli* lysates before (NI) and after (I) induction, isolated inclusion bodies (IB), and purified subunits (HQ). BL21 DE3 *E. coli* was previously transformed with either pLMI DR $\alpha_{\text{cys}}$  (lanes 1–4) or pLMI DR $\beta$  (lanes 5–8). (b) SDS-PAGE of DR1-peptide-biotin complexes. Each section consist of equivalents amounts of DR1-peptide $_{\text{cys}}$ -biotin that had not been boiled, boiled for 2 min, or boiled, chilled, and supplemented with excess SA. Efficient peptide loading is indicated by the migration of  $\alpha\beta$  heterodimer (DR-pep $_{\text{bio}}$ ) complexes in the not-boiled lanes. Efficient biotinylation is demonstrated by the disappearance of  $\alpha_{\text{bio}}$  after the addition of SA and the formation of SA- $\alpha_{\text{bio}}$ . Biotinylation can also be observed for the  $\alpha_{\text{cys}}$  constructs by the small gel shift between  $\alpha_{\text{cys}}$  and  $\alpha_{\text{bio}}$  bands. The first lane contains DR $\alpha_{\text{cys}}$  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.

intact complexes (– boil). The absence of free  $\alpha$  or  $\beta$  subunits from the not 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. 3). Biotinylation of DR $\alpha_{\text{cys}}$  can be observed by a slight shift in the mobility of the subunit in gel electrophoresis (Fig. 2b, compare DR $\alpha_{\text{cys}}$  to the DR $\alpha_{\text{bio}}$  band of boiled DR-pep $_{\text{cys}}$  complexes). However, biotinylation is more clearly demonstrated by the supershift of DR $\alpha_{\text{bio}}$  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).

### 3.2. 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 and 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. 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 (Stern and Wiley, 1992; Kozono et al., 1994). 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 $_{\text{cys}}$  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

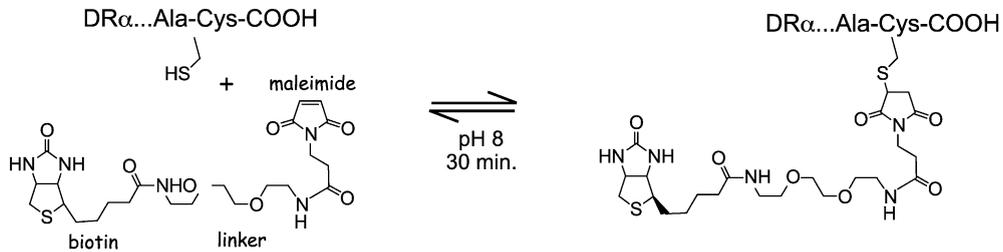
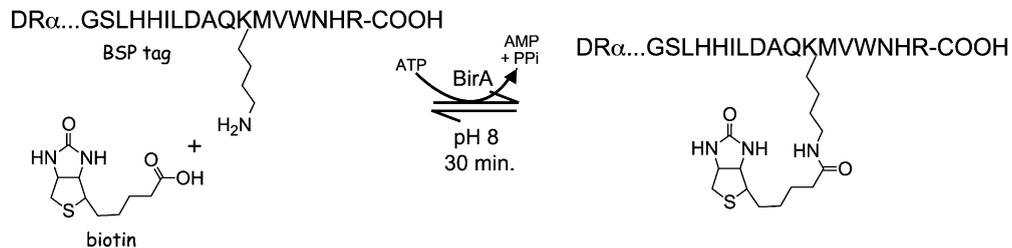
**a Chemical biotinylation****b Enzymatic biotinylation**

Fig. 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.

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. 4a), biotinylated, and used to stain T cells with comparable brightness to DR–peptide complexes produced in *E. coli* (Fig. 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.

### 3.3. 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<sup>+</sup> T cells (compare Fig. 5a vs. b). Although PE is a brighter fluorophore than FITC, the difference was greater than the relative fluorescent efficiencies intrinsic

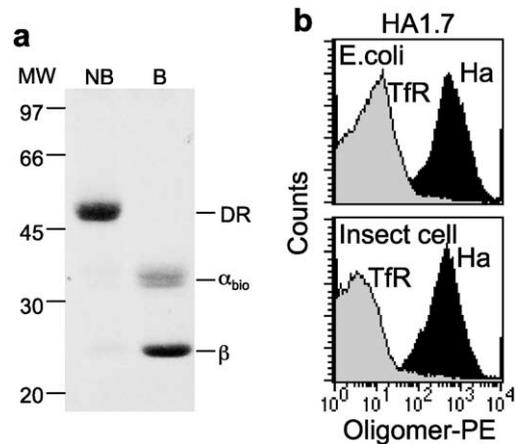


Fig. 4. DR1–peptide complexes produced in insect cells. (a) As an alternative to expression in *E. coli*, DR1<sub>cys</sub> was expressed in S2 insect cells, purified, biotinylated, loaded with Ha peptide and analyzed by SDS-PAGE as not boiled (NB) or boiled (B). Quantitative peptide loading is indicated 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 37 °C with SA–PE oligomers of DR1–Ha (black) or DR1–TfR (gray) from *E. coli* (upper panel), or from S2 cells (lower panel).

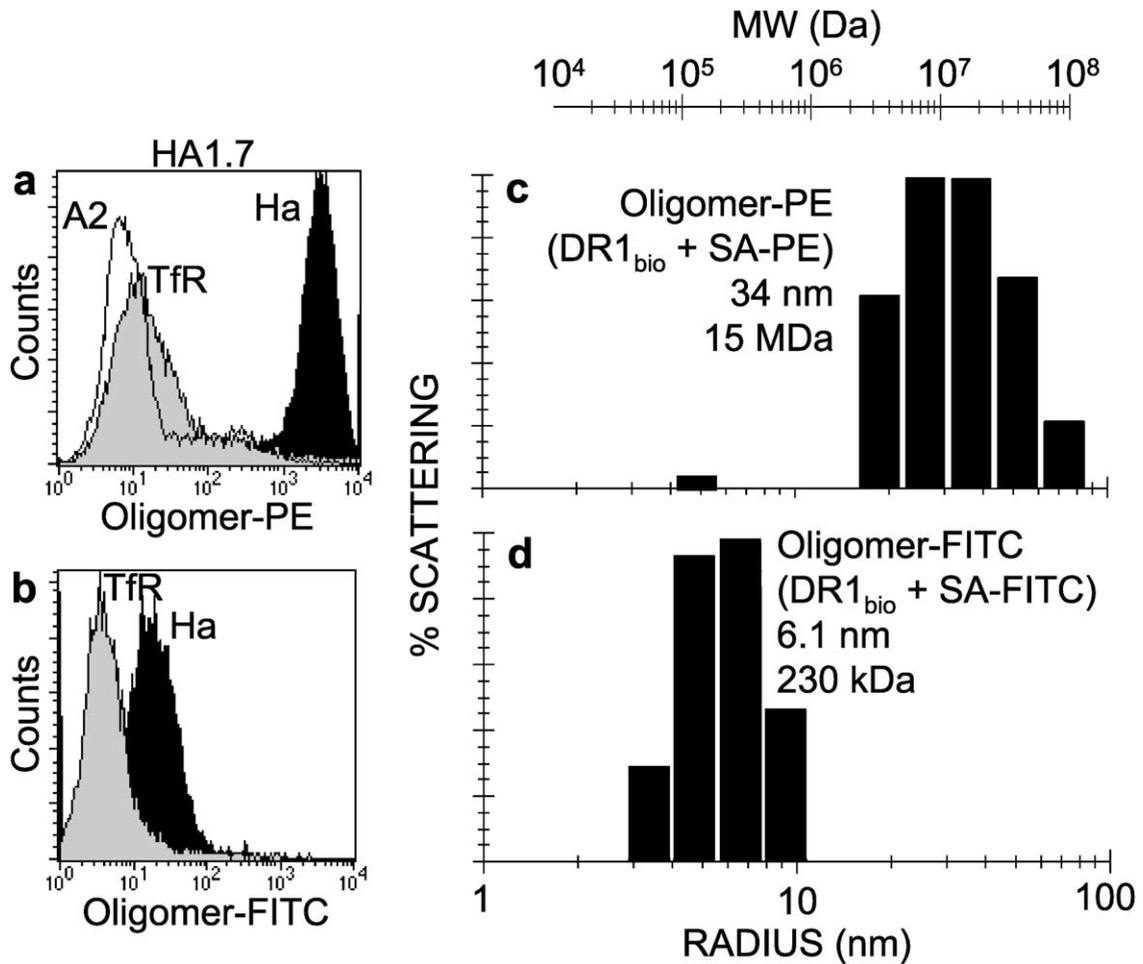


Fig. 5. Comparison of DR oligomers formed with SA-PE and SA-FITC. (a, b) Comparison of the staining of the different oligomers. HA1.7 T cells were stained at 37 °C with SA-PE (a) or SA-FITC (b) oligomers of DR1-Ha (black), DR1-A2 (unfilled), or DR1-TfR (gray). DR1-Ha oligomers formed with SA-PE (a) were 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.

sis 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 cross-linking. In our experience, commercially available SA-PE preparations, even those with average SA/PE ratios of 1:1, contain large cross-linked 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. 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. 5d). In contrast, DR1 oligomerized with SA–FITC (Fig. 5c) 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 appear to be due in part to SA cross-linking, in addition to the intrinsic brightness of the PE fluorophore. This point is under-appreciated by many 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. Fig. 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. 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 and Stern, in press).

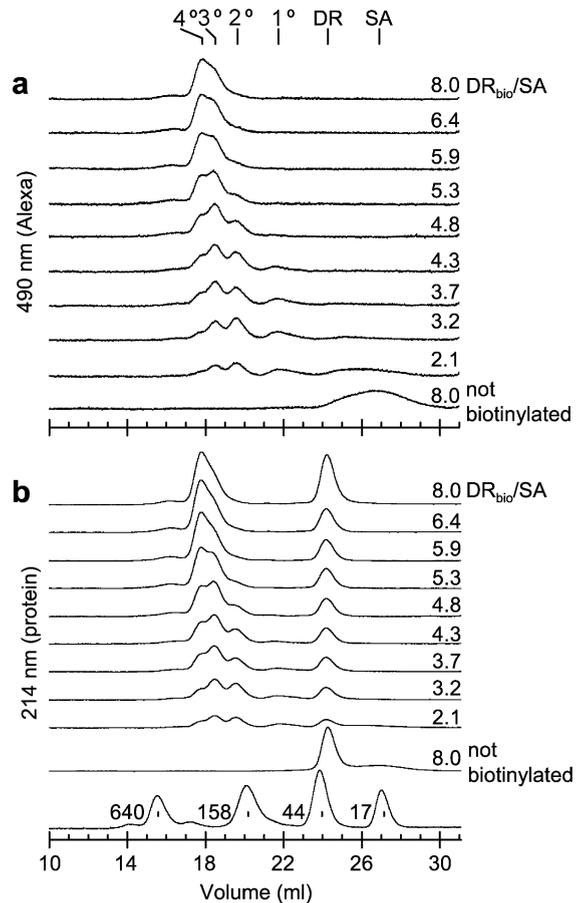


Fig. 6. Oligomerization of DR1– $\text{pep}_{\text{bio}}$  by SA–Alexa. Gel filtration chromatography was performed on mixtures of DR1–TfR–biotin and SA–Alexa, and monitored at (a) 490 nm and (b) 214 nm, using two columns in series. Molar ratios of DR to SA are indicated at the right of each trace. The 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.

### 3.4. 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-restricted, 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. 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 nonspecific staining by the DR1 oligomers, and can be seen as the large CD4<sup>mid</sup> population in the center of the profiles shown in Fig. 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 (Pharminogen) 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 nonspecific staining carefully in each experiment, particularly if low-frequency populations are under investigation. This can be accom-

plished 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 II 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

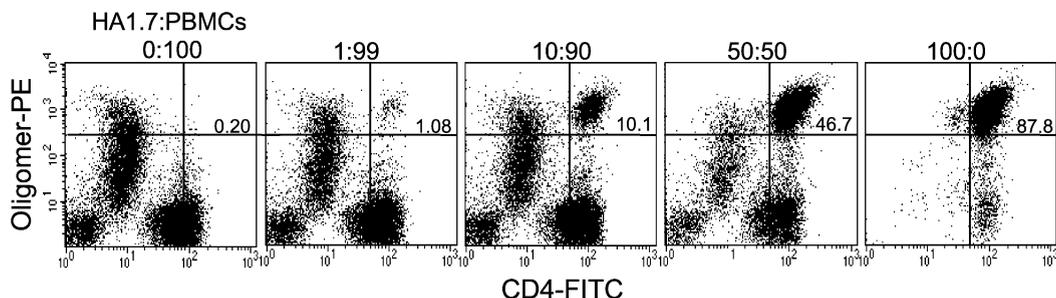


Fig. 7. Determination of detection limit in mixed lymphocyte populations. In order to assess the potential for detection of antigen-specific CD4<sup>+</sup> T cells in fresh PBMC samples, a clone of known specificity (HA1.7) was diluted into nonspecific PBMCs at the ratios shown above each plot. Cells were stained with SA-PE oligomers of DR1-Ha at 37 °C, co-stained with  $\alpha$ -CD4-FITC at 4 °C, and analyzed by flow cytometry. Percentage of cells CD4<sup>+</sup> PE<sup>+</sup> is indicated in the upper-right quadrant of each plot. Specific CD4<sup>+</sup> T cells were readily detected at a 1:99 frequency, but detection below this limit may be difficult. Monocytes, identified by CD4<sup>low</sup> and FSC<sup>large</sup> phenotype and observed in the center of the plots, bound nonspecifically 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. This figure taken from Cameron et al. (2001).

perfect measure of the nonspecific staining, we suggest that both be examined.

Many antigen-specific CD4<sup>+</sup> 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<sup>+</sup> T cells in peripheral lymphocytes using class II MHC oligomers. Meyer et al. (2000) were able to detect DR4-restricted, OspA-peptide-specific T cells ex vivo using DR4 oligomers. 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<sup>+</sup> 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 autoimmune states.

### 3.5. In vitro expansion of antigen-specific CD4<sup>+</sup> T cells and detection by MHC oligomers

Novak et al. (1999) 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. CFSE is a non-specific amine-reactive fluorescein derivative that is stably incorporated into cells and whose signal is diluted twofold with each cell division. Using this method, we were able to detect the responsive Ha-specific CD4<sup>+</sup> T cell populations from two DR1 individuals seven days after initial in vitro stimulation (Fig. 8a,b). Cells in the two left quadrants have divided since the initial stimulation (CFSE<sup>low</sup>). Of these, the oligomer-PE<sup>+</sup> 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

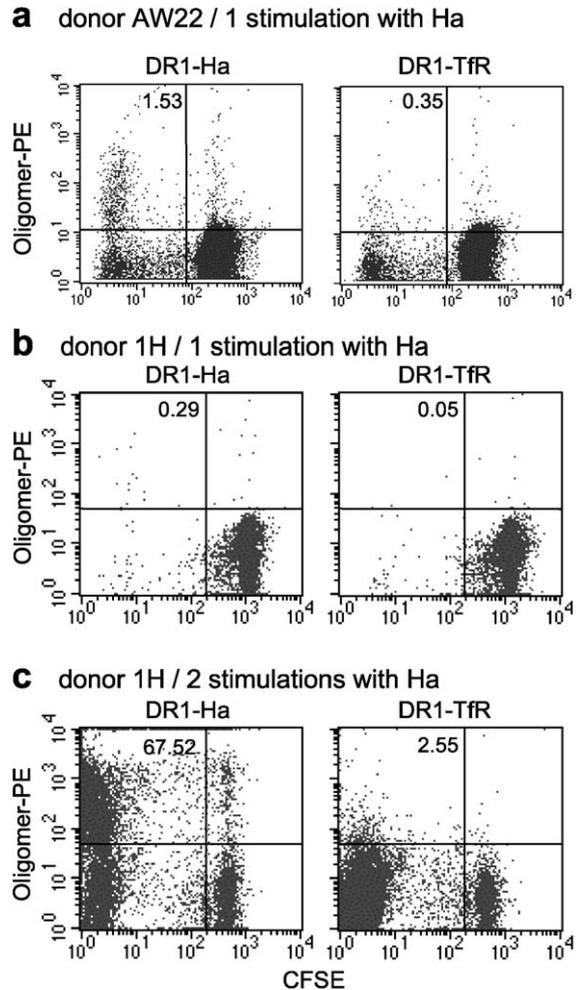


Fig. 8. Short-term in vitro expansion of PBMCs to detect antigen-specific CD4<sup>+</sup> T cells. Since Ha-specific CD4<sup>+</sup> T cells were undetectable in the fresh PBMCs of multiple donors (data not shown), cells were expanded by in vitro stimulation with Ha peptide. (a, b) After one stimulation, Ha-specific T cells could be detected. (c) After a second stimulation, the culture was dominated by Ha-specific CD4<sup>+</sup> T cells in the CFSE<sup>low</sup>, Oligomer-PE<sup>+</sup> 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  $\mu$ M Ha, and stained 7 days later with DR1–Ha or DR1–TfR at 37  $^{\circ}$ C. The 1H culture was stimulated a second time by 5  $\mu$ M Ha presented by DR1 EBV B cells, and stained 11 days later with oligomers of DR1–Ha or DR1–TfR at 37  $^{\circ}$ C. Each plot was gated for live, CD4<sup>+</sup> 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<sup>low</sup> (proliferating), PE<sup>+</sup> (antigen-specific) cells is indicated in the upper left quadrant of each plot.

CFSE signal, indicating that they underwent six or more divisions (the CFSE<sup>low</sup> signal is close to the autofluorescence level and cannot be accurately measured). Very few CFSE<sup>low</sup> cells were observed in samples stimulated with control peptides (data not shown). An upper-limit for the frequency of the DR1–Ha specific CD4<sup>+</sup> 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<sup>low</sup>, oligomer-PE<sup>+</sup> population was observed to dominate the culture after 11 days (Fig. 8c).

### 3.6. 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 h at either 4, 22, or 37 °C (Fig. 9). The clone HA1.7 showed no detectable staining at 4 °C (Fig. 9a), and the clone CI-1 shows slight staining at 4 °C (Fig. 9b). In contrast, another DR1–Ha specific clone, HaCOH8 (Fig. 9c), and a DR1–p24(34) specific clone, AC-25-1 (Fig. 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

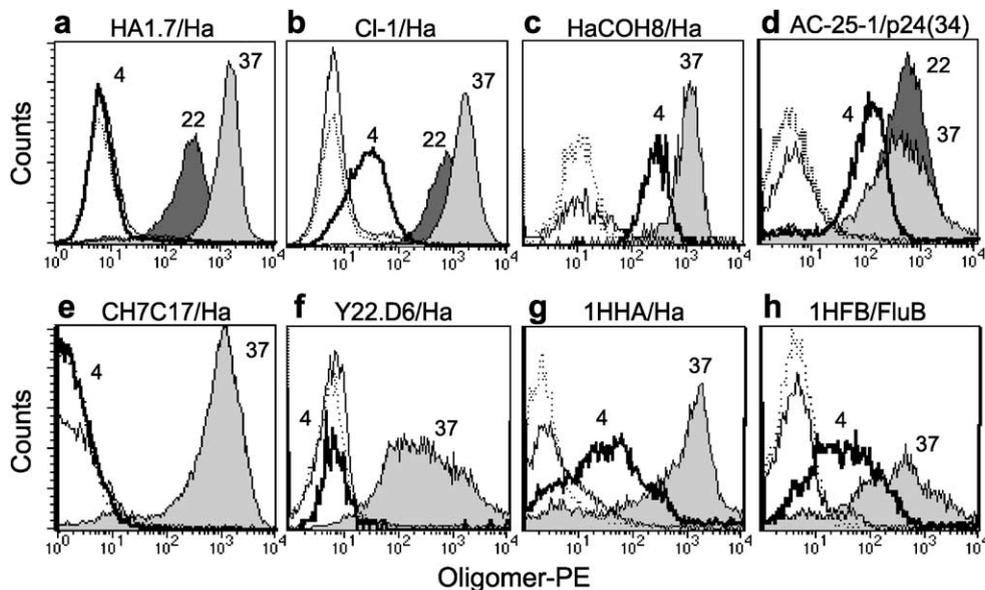


Fig. 9. Oligomer staining of T cell clones, transfectants, and short-term polyclonal lines, at different temperatures. Some CD4<sup>+</sup> T cells can be detected only when stained at 37 °C, while others can be readily identified at either 4 or 37 °C. (a–h) Cells were stained at 37 °C with SA–PE oligomers of DR1–Ha (shaded trace) or DR1–TfR (thin trace) or at 4 °C with SA–PE oligomers of DR1–Ha (thick trace) or DR1–TfR (dashed trace). Selected clones were also stained with DR1–Ha oligomers at 22 °C (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<sup>+</sup> PMBCs against either FluB (g) or Ha (h) peptide.

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. 9e), and for RBL mast cells similarly transfected (Y22.D6, Fig. 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.

### 3.7. Short-term polyclonal T cell lines show heterogeneous staining at 4 °C

The polyclonal lines IHFB and IHHA 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<sup>+</sup> 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. 9g,h, 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<sup>+</sup> T cells exhibiting different temperature dependence for class II MHC oligomer staining also exist in vivo at significant frequencies.

## 4. Discussion

### 4.1. 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<sup>k</sup> (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 (<http://www.niaid.nih.gov/reposit/tetramer/index.html>), which currently produces 16 human, 8 murine, 6 macaque and 2 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 and Wiley, 1992), DR4 (B1\*0401) (Kozono et al., 1994), DR52a (B3\*0101) (Gorski, personal communication), I–E<sup>k</sup> (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 (Gauthier et al., 1998; Appel et al., 2000), I–A<sup>d</sup> (Rhode et al., 1996; Scott et al., 1996), I–A<sup>g7</sup> (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<sup>+</sup> 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.

#### 4.2. 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 Fig. 8, each T cell expansion contains some CFSE<sup>low</sup> 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<sup>+</sup> T cells do not 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 that while many CD4<sup>+</sup> 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<sup>+</sup> T cells, there have also been several reports of CD8<sup>+</sup> T cells specific for particular peptides which could not be stained by cognate MHC oligomers (de Visser et al., 2000; Spencer and Braciale, 2000; Reignat et al., 2002; Moser et al., 2001). 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<sup>+</sup> T cells (Goulder et al., 2000; Welsh, 2001). Considering the relative paucity of investigations of CD4<sup>+</sup> T cells using class II MHC oligomers, we cannot yet be certain what types of cells will and will not be detectable by class II MHC oligomers. This should continue to be a focus for researchers in the field.

#### 4.3. 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 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<sup>+</sup> 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. 9). For both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, elevated temperatures have been shown to facilitate the internalization of MHC oligomers (Whelan et al., 1999; Cameron et al., 2001). 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 (Garcia et al., 1996; Ding et al., 1999). In this case, enthalpic terms would be expected to dominate the affinity and to favor

tighter binding and lower temperatures. However, Willcox et al. (1999) found evidence for a large entropic factor in the binding of two different class I MHC/TCR pairs. 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 and 1.8  $\mu\text{M}$ , 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 cross-linking. We have previously used a model for this reaction that parameterizes avidity as the dissociation constant,  $K_d$ , and a cross-linking or oligomerization constant,  $K_x$  (Perelson and Delisi, 1980). Using this model, we determined that HA1.7 and HaCOH8, although they had similar  $K_d$ 's, differed in their  $K_x$ 's by more than 10-fold, with HaCOH8 being the “easier to cross link” clone (Stone et al., 2001). The increased cross-linkability (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 cross-linkability 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 et al. (2001) 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. They observed higher  $K_x$  values for the memory cells, suggesting a cross-linkability 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<sup>+</sup> T cells (de Visser et al., 2000; Hesse et al., 2001; Margulies, 2001; Slifka and Whitton, 2001). The short-term polyclonal CD4<sup>+</sup> T cell lines studied here contain cells of high, low, and intermediate avidity. Avidity modulation may be an important way for the immune system to regulate 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 individual clones or their varied activation states.

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